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(54) **Biosynthetic osteogenic proteins and osteogenic devices containing them**

Biosynthetische knochenbildende Proteine, und sie enthaltende knochenbildende Vorrichtungen

Protéines biosynthétiques ostéogènes, et dispositifs contenant les-dites

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- **ÖZKAYNAK, E. ET AL.: "OP-1 cDNA encodes an osteogenic protein in the TGF-beta family" EMBO J., vol. 9, no. 7, 1990, page 2085-2093 XP000611252**
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Description

[0001] This invention relates to osteogenic devices, to synthetic genes encoding proteins which can induce osteogenesis in mammals and methods for their production using recombinant DNA techniques, to synthetic forms of osteogenic protein, and to bone and cartilage repair procedures using osteogenic device comprising the synthetic proteins.

[0002] Mammalian bone tissue is known to contain one or more proteinaceous materials, presumably active during growth and natural bone healing, which can induce a developmental cascade of cellular events resulting in endochondral bone formation. This active factor (or factors) has variously been referred to in the literature as bone morphogenetic or morphogenic protein, bone inductive protein, osteogenic protein, osteogenin, or osteoinductive protein.

[0003] The developmental cascade of bone differentiation consists of recruitment of mesenchymal cells, proliferation of progenitor cells, calcification of cartilage, vascular invasion, bone formation, remodeling, and finally marrow differentiation (Reddi (1981) Collagen Rel. Res. 1:209-226).

[0004] Though the precise mechanisms underlying these phenotypic transformations are unclear, it has been shown that the natural endochondral bone differentiation activity of bone matrix can be dissociatively extracted and reconstituted with inactive residual collagenous matrix to restore full bone induction activity (Sampath and Reddi, (1981) Proc. Natl. Acad. Sci. USA 78:7599-7603). This provides an experimental method for assaying protein extracts for their ability to induce endochondral bone in vivo.

[0005] This putative bone inductive protein has been shown to have a molecular mass of less than 50 kilodaltons (kD). Several species of mammals produce closely related protein as demonstrated by cross species implant experiments (Sampath and Reddi (1983) Proc. Natl. Acad. Sci. USA 80:6591-6595).

[0006] The potential utility of these proteins has been widely recognized. It is contemplated that the availability of the pure protein would revolutionize orthopedic medicine, certain types of plastic surgery, and various periodontal and craniofacial reconstructive procedures.

[0007] The observed properties of these protein fractions have induced an intense research effort in various laboratories directed to isolating and identifying the pure factor or factors responsible for osteogenic activity. The current state of the art of purification of osteogenic protein from mammalian bone is disclosed by Sampath et al. (Proc. Natl. Acad. Sci. USA (1987) 80). Urist et al. (Proc. Soc. Exp. Biol. Med. (1984) 173:194-199) disclose a human osteogenic protein fraction which was extracted from demineralized cortical bone by means of a calcium chloride-urea inorganic-organic solvent mixture, and retrieved by differential precipitation in guanidine-hydrochloride and preparative gel electrophoresis. The authors report that the protein fraction has an amino acid composition of an acidic polypeptide and a molecular weight in a range of 17-18 kD.

[0008] Urist et al. (Proc. Natl. Acad. Sci. USA (1984) 81:371-375) disclose a bovine bone morphogenetic protein extract having the properties of an acidic polypeptide and a molecular weight of approximately 18 kD. The authors reported that the protein was present in a fraction separated by hydroxyapatite chromatography, and that it induced bone formation in mouse hindquarter muscle and bone regeneration in trephine defects in rat and dog skulls. Their method of obtaining the extract from bone results in ill-defined and impure preparations.

[0009] European Patent Application Serial No. 148,155, published October 7, 1985, purports to disclose osteogenic proteins derived from bovine, porcine, and human origin. One of the proteins, designated by the inventors as a P3 protein having a molecular weight of 22-24 kD, is said to have been purified to an essentially homogeneous state. This material is reported to induce bone formation when implanted into animals.

[0010] International Application No. PCT/087/01537, published January 14, 1988, discloses an impure fraction from bovine bone which has bone induction qualities. The named applicants also disclose putative bone inductive factors produced by recombinant DNA techniques. Four DNA sequences were retrieved from human or bovine genomic or cDNA libraries and apparently expressed in recombinant host cells. While the applicants stated that the expressed proteins may be bone morphogenetic proteins, bone induction was not demonstrated. See also Urist et al., EP 0,212,474 entitled Bone Morphogenic Agents.

[0011] Wang et al. (Proc. Nat. Acad. Sci. USA (1988) 85: 9484-9488) discloses the purification of a bovine bone morphogenetic protein from guanidine extracts of demineralized bone having cartilage and bone formation activity as a basic protein corresponding to a molecular weight of 30 kD determined from gel elution. Purification of the protein yielded proteins of 30, 18 and 16 kD which, upon separation, were inactive. In view of this result, the authors acknowledged that the exact identity of the active material had not been determined.

[0012] Wozney et al. (Science (1988) 242: 1528-1534) discloses the isolation of full-length cDNA's encoding the human equivalents of three polypeptides originally purified from bovine bone. The authors report that each of the three recombinantly expressed human proteins are independently or in combination capable of inducing cartilage formation. No evidence of bone formation is reported.

[0013] It is an object of this invention to provide osteogenic devices comprising matrices containing dispersed osteogenic protein capable of bone induction in allogenic and xenogenic implants. Another object is to provide synthetic osteogenic proteins capable of inducing endochondral bone formation in mammals, including humans. Yet another

object is to provide genes encoding non-native osteogenic proteins and methods for their production using recombinant DNA techniques. Another object is to provide novel biosynthetic forms of osteogenic proteins and a structural design for novel, functional osteogenic proteins. Another object is to provide methods for inducing cartilage formation.

[0014] These and other objects and features of the invention will be apparent from the description, drawings, and claims which follow.

Summary of the Invention

[0015] This invention involves osteogenic devices which, when implanted in a mammalian body, can induce at the locus of the implant the full developmental cascade of endochondral bone formation and bone marrow differentiation. Suitably modified as disclosed herein, the devices also may be used to induce cartilage formation. The devices comprise a carrier material, referred to herein as a matrix, having the characteristics disclosed below, containing dispersed osteogenic protein in the form of a biosynthetic construct.

[0016] Key to these developments was the successful preparation of substantially pure osteogenic protein by purification from bone, the elucidation of amino acid sequence and structure data of the native osteogenic protein, and insights involving study of the DNA and amino acid sequences of the natural source product. A protocol was developed which results in retrieval of active, substantially pure osteogenic protein from mammalian bone. Investigation of the properties and structure of the native form osteogenic protein then permitted the inventors to develop a rational design for non-native forms, i.e., forms never before known in nature, capable of inducing bone formation. As far as applicants are aware, the constructs disclosed herein constitute the first instance of the design of a functional, active protein without preexisting knowledge of the active region of a native form nucleotide or amino acid sequence.

[0017] A series of consensus DNA sequences were designed with the goal of producing an active osteogenic protein. The sequences were based on partial amino acid sequence data obtained from the naturally sourced product and on observed homologies with unrelated genes reported in the literature, or the sequences they encode, having a presumed or demonstrated developmental function. Several of the biosynthetic consensus sequences have been expressed as fusion proteins in procaryotes, purified, cleaved, refolded, combined with a matrix, implanted in an established animal model, and shown to have endochondral bone-inducing activity. The currently preferred active proteins comprise sequences designated COPS, COP7, COP16, and OP1. The amino acid sequences of these proteins are set forth below.

```

      1      10      20      30      40
COP5  LYVDFS-DVGWDDWIVAPPGYQAFYCHGECPFPLAD
      50      60      70
      HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
      80      90     100
      ISMLYLDENEKVVVLKQEMVVEGCGCR

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      1      10      20      30      40
COP7  LYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
      50      60      70
      HLNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
      80      90     100
      ISMLYLDENEKVVVLKQEMVVEGCGCR

```


1 10 20 30 40 50
 CXXXXLXVFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXNHAIX
 5 60 70 80 90 100
 QXXVXXNXXXXPXXCCXPXXXXXXLXXXXXXVXLXXYXXMXVXXCXCX

where the letters indicate the amino acid residues of standard single letter code, and the Xs each represent any one of the 22 naturally occurring amino acid residues. Preferred amino acid sequences within the foregoing generic sequences are:

1 10 20 30 40 50
 LYVDFRDVGWNDWIVAPPGYHAFYCHGECPPFLADHLNSTNHAIV
 K S S L QE VIS E FD Y E A AY MPESMKAS VI
 F E K I DN L N S Q ITK F P TL
 A S K
 60 70 80 90 100
 QTLVNSVNPFGKIPKACCVPTELSAISMLYLDENENVVLKNYQDMVVEGCGCR
 SI HAI SEQV EP A EQMNSLAI FFNDQDK I RK EE T DA H H
 RF T S K DPV V Y N S H RN RS
 N S K P E

and

1 10 20 30 40 50
 CKRHPLYVDFRDVGWNDWIVAPPGYHAFYCHGECPPFLADHLNSTNHAIV
 RRRS K S S L QE VIS E FD Y E A AY MPESMKAS VI
 KE F E K I DN L N S Q ITK F P TL
 Q A S K
 60 70 80 90 100
 QTLVNSVNPFGKIPKACCVPTELSAISMLYLDENENVVLKNYQDMVVEGCGCR
 SI HAI SEQV EP A EQMNSLAI FFNDQDK I RK EE T DA H H
 RF T S K DPV V Y N S H RN RS
 N S K P E

wherein each of the amino acids arranged vertically at each position in the sequence may be used alternatively in various combinations. Note that these generic sequences have 6 and preferably 7 cysteine residues where inter- or intramolecular disulfide bonds can form, and contain other critical amino acids which influence the tertiary structure of the proteins. These generic structural features are found in previously published sequences, none of which have been described as capable of osteogenic activity, and most of which never have been linked with such activity.

[0021] Particular useful sequences include:

Vg1 1 10 20 30 40
 CKKRHLYVEFK-DVGWQNWVIAPOGYMANCYGECPYPLTE
 5 50 60 70
 ILNGSN--H-AILQTLVHSIEPED-IPLPCCVPTKMSP
 80 90 100
 ISMLFYDNNDNVVLRHYENMAVDECGCR

DPP 1 10 20 30 40
 CRRHSLYVDFS-DVGWDDWIVAPLGDAYYCHGKCPFPLAD
 15 50 60 70
 HFNSTN--H-AVVQTLVNNNNPGK-VPKACCVPTQLDS
 80 90 100
 VAMLYLNDQSTVVVLKNYQEMTVVGCGCR

CBMP-2a 1 10 20 30 40
 CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPPFLAD
 25 50 60 70
 HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
 80 90 100
 ISMLYLDENEKVVLKNYQDMVVEGCGCR

CBMP-2b 1 10 20 30 40
 CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
 35 50 60 70
 HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
 80 90 100
 ISMLYLDEYDKVVLKNYQEMVVEGCGCR

CBMP-3 1 10 20 30 40
 CARRYLKVDFE-DIGWSEWIIISPKSFDAYYCSGACQFPMPK
 45 50 60 70
 SLKPSN--H-ATIQSIVRAVGVPVPGIPEPCCVPEKMSS
 80 90 100
 LSILFFDENKNVVVLKVYPNMTVESACR

COP1 1 10 20 30 40
 LYVDFQRDVGWDDWIIAPVDFDAYYCSGACQFPSAD
 50 60 70
 HFNSTN--H-AVVQTLVNNMNPBK-VPKPCCVPTELSA
 55 80 90 100
 ISMLYLDENSTVVVLKNYQEMTVVGCGCR

COP4

1 10 20 30 40
LYVDFS-DVGWDDWIVAPPGYQAFYCSGACQFPSAD
50 60 70
HFNSTN--H-AVVQTLVNMMNPGK-VPKPCCVPTELSA
80 90 100
ISMLYLDENEKVVLKNYQEMVVEGCGR

[0025] The osteogenic proteins and implantable osteogenic devices enabled and disclosed herein will permit the physician to obtain optimal predictable bone formation to correct, for example, acquired and congenital craniofacial

and other skeletal or dental anomalies (Glowacki et al. (1981) Lancet 1:959-963). The devices may be used to induce local endochondral bone formation in non-union fractures, and in other clinical applications including periodontal applications where bone formation is required. Another potential clinical application is in cartilage repair, for example, in the treatment of osteoarthritis.

Brief Description of the Drawing

[0026] The foregoing and other objects of this invention, the various features thereof, as well as the invention itself, may be more fully understood from the following description, when read together with the accompanying drawings, in which:

FIGURE 1 is a comparison of the amino acid sequence of various osteogenic proteins to those of the TGF-beta family. COP1, COP3, COP4, COPS, and COP7 are a family of analogs of synthetic osteogenic proteins developed from the consensus gene that was joined to a leader protein via a hinge region having the sequence D-P-N-G that permitted chemical cleavage at the D-P site (by acid) or N-G (by hydroxylamine) resulting in the release of the analog protein; VGI is a *Xenopus* protein, DPP is a *Drosophila* protein; OP1 is a native osteogenic protein; CBMP2a and 2b, and CBMP3 are subparts of proteins disclosed in PCT application 087/01537; MIS is Mullerian inhibitory substance; and "consensus choices" represent various substitutions of amino acids that may be made at various positions in osteogenic proteins;

FIGURE 2A is an *E. coli* expression vector containing a gene of an osteogenic protein fused to a leader protein;

FIGURE 2B is the DNA sequence comprising a modified trp-LE leader, two Fb domains of protein A, an ASP-PRO cleavage site, and the COPS sequence;

FIGURES 3A and 3B are photomicrographs of implants showing the histology (day 12) of COPS active recombinant protein. A is control (rat matrix alone, 25 mg). B is rat matrix plus COP5, showing +++ cartilage formation and ++ bone formation (see key infra). Similar results are achieved with COP7; and

FIGURE 4 is a schematic representation of the DNA sequence and corresponding amino acid sequence of a consensus gene for osteogenic protein (COPO).

Description

[0027] Purification protocols have been developed which enable isolation of the osteogenic protein present in crude protein extracts from mammalian bone. The isolation procedure enables the production of significant quantities of substantially pure osteogenic protein from any mammalian species, provided sufficient amounts of fresh bone from the species is available. The empirical development of the procedure, coupled with the availability of fresh calf bone, enabled isolation of substantially pure bovine osteogenic protein (BOP). BOP has been characterized significantly; its ability to induce cartilage and ultimately endochondral bone growth in cat, rabbit, and rat have been studied; it has been shown to be able to induce the full developmental cascade of bone formation previously ascribed to unknown protein or proteins in heterogeneous bone extracts; and it may be used to induce formation of endochondral bone in orthopedic defects including non-union fractures. In its native form it is a glycosylated, dimeric protein. However, it is active in deglycosylated form. It has been partially sequenced.

[0028] Elucidation of the amino acid sequence of BOP enabled the construction of a consensus nucleic acid sequence designed as disclosed herein based on the sequence data, inferred codons for the sequences, and observation of partial homology with known genes.

[0029] These consensus sequences have been refined by comparison with the sequences present in certain regulatory genes from *drosophila*, *xenopus*, and human followed by point mutation, expression, and assay for activity. This approach has been successful in producing several active totally synthetic constructs not found in nature (as far as applicants are aware) which have true osteogenic activity.

[0030] These discoveries enable the construction of DNAs encoding totally novel, non-native protein constructs which individually and combined are capable of producing true endochondral bone. The DNAs may be expressed using well established recombinant DNA technologies in procaryotic or eucaryotic host cells, and the expressed proteins may be oxidized and refolded *in vitro* if necessary for biological activity.

[0031] The design and production of such biosynthetic proteins, the nature of the matrix, and other material aspects concerning the nature, utility, how to make, and how to use the subject matter claimed herein will be further understood from the following, which constitutes the best method currently known for practicing the various aspects of the invention.

CONSENSUS SEQUENCE DESIGN

[0032] A synthetic consensus gene shown in FIGURE 4 was designed to encode a consensus protein based on amino acid predictions from homology with the TGF-beta gene family. The designed consensus sequence was then constructed using known techniques involving assembly of oligonucleotides manufactured in a DNA synthesizer.

[0033] Tryptic peptides derived from Bovine Osteogenic Protein isolated by applicants and sequenced by Edman degradation provided amino acid sequences that showed strong homology with the Drosophila DPP protein sequence (as inferred from the gene), the Xenopus VG1 protein, and somewhat less homology to inhibin and TGF-beta, as demonstrated below in TABLE 1.

TABLE 1

<u>protein</u>	<u>amino acid sequence</u>	<u>homology</u>
(<u>BOP</u>)	SFDAYYCSGACQFPS ***** * * *	(9/15 matches)
(<u>DPP</u>)	GYDAYYCHGKCPFFL	
(<u>BOP</u>)	SFDAYYCSGACQFPS * * * * *	(6/15 matches)
(<u>Vq1</u>)	GYMANYCYGECPYPL	
(<u>BOP</u>)	SFDAYYCSGACQFPS * * * * *	(5/15 matches)
(<u>inhibin</u>)	GYHANYCEGECPSHI	
(<u>BOP</u>)	SFDAYYCSGACQFPS * * * * *	(4/15 matches)
(<u>TGF-beta</u>)	GYHANFCLGPCPYIW	
(<u>BOP</u>)	K/RACCVPTELSAISMLYLDEN ***** * * * * *	(12/20 matches)
(<u>Vq1</u>)	LPCCVPTKMSPISMLFYDNN	
(<u>BOP</u>)	K/RACCVPTELSAISMLYLDEN * * * * * * * * * *	(12/20 matches)
(<u>inhibin</u>)	KSCCVPTKLRPMSMLYYDDG	
(<u>BOP</u>)	K/RACCVPTELSAISMLYLDE ***** * *	(6/19 matches)
(<u>TGF-beta</u>)	APCCVPQALEPLPIVYYVG	
(<u>BOP</u>)	K/RACCVPTELSAISMLYLDEN ***** * * * *	(12/20 matches)
(<u>DPP</u>)	KACCVPTQLDSVAMLYLNDQ	

(BOP) LYVDF

 (DPP) LYVDF (5/5 matches)

(BOP) LYVDF
 *** *
 (Vgl) LYVEF (4/5 matches)

(BOP) LYVDF
 ** **
 (TGF-beta) LYIDF (4/5 matches)

(BOP) LYVDF
 * *
 (inhibin) FFVSF (2/5 matches)

*-match

[0034] In determining an appropriate amino acid sequence of an osteogenic protein (from which the nucleic acid sequence can be determined), the following points were considered: (1) the amino acid sequence determined by Edman degradation of natural source osteogenic protein tryptic fragments is ranked highest as long as it has a strong signal and shows homology or conservative changes when aligned with the other members of the gene family; (2) where the sequence matches for all four proteins, it is used in the synthetic gene sequence; (3) matching amino acids in DPP and Vgl are used; (4) If Vgl or DPP diverged but either one were matched by inhibin or by TGF-beta, this matched amino acid is chosen; (5) where all sequences diverged, the DPP sequence is initially chosen, with a later plan of creating the Vgl sequence by mutagenesis kept as a possibility. In addition, the consensus sequence is designed to preserve the disulfide crosslinking and the apparent structural homology among the related proteins.

RECOMBINANT OSTEOGENIC PROTEIN CONSTRUCTS

[0035] This approach resulted in the production of novel recombinant proteins capable of inducing formation of cartilage and endochondral bone comprising a protein structure analogous to or duplicative of the functional domain of the naturally sourced material. The amino acid sequences encoded by the consensus DNA sequences were derived from a family of natural proteins implicated in tissue development. These gene products/proteins are known to exist in active form as dimers and are, in general, processed from a precursor protein to produce an active C-terminal domain of the precursor.

[0036] The recombinant osteogenic/chondrogenic proteins are "novel" in the sense that, as far as applicants are aware, they do not exist in nature or, if they do exist, have never before been associated with bone or cartilage formation. The approach to design of these proteins is to employ amino acid sequences, found in the native OP isolates, in polypeptide structures are patterned after certain proteins reported in the literature, or the amino acid sequences inferred from DNAs reported in the literature. Thus, using the design criteria set forth above, and refining the amino acid sequence as more protein sequence information was learned, a series of synthetic proteins were designed with the hope and intent that they might have osteogenic or chondrogenic activity when tested in the bioassay system disclosed below.

[0037] It was noted, for example, that DPP from drosophila, VG1 from *Xenopus*, the TGF beta family of proteins, and to a lesser extent, alpha and beta inhibins, had significant homologies with certain of the sequences derived from

the naturally sourced OP product. (FIGURE 1.) Study of these proteins led to the realization that a portion of the sequence of each had a structural similarity observable by analysis of the positional relationship of cysteines and other amino acids which have an important influence on three dimensional protein conformation. It was noted that a region of these sequences had a series of seven cysteines, placed very nearly in the same relative positions, and certain other amino acids in sequence as set forth below:

```

      10      20      30      40      50
CXXXLXVFXDXGWXXWXXXPXGXXAXYCXGXCCXPXXXXXXNHAXX
      60      70      80      90     100
QXXVXXXNXXXXPXXCCXPXXXXXXXXLXXXXXXXXVXLXXYXXMXVXXCXCX

```

wherein each X independently represents an amino acid. Expression experiments of two of these constructs demonstrate activity. Expression experiments with constructs patterned after this template amino acid sequence with a shorter sequence having only six cysteines also show activity:

```

      10      20      30      40      50
LXVFXDXGWXXWXXXPXGXXAXYCXGXCCXPXXXXXXNHAXX
      60      70      80      90     100
QXXVXXXNXXXXPXXCCXPXXXXXXXXLXXXXXXXXVXLXXYXXMXVXXCXCX

```

wherein each X independently represents an amino acid. Within these generic structures are a multiplicity of specific sequences which have osteogenic or chondrogenic activity. Preferred structures are those having the amino acid sequence:

```

      10      20      30      40      50
CKRHPLYVDFRDVGWNDWIVAPPGYHAFYCHGECPPFLADHLNSTNHAIV
RKRS K S S L QE VIS E FD Y E A AY MPESMKAS VI
KE F E K I DN L N S Q ITK F P TL
Q A S K
      60      70      80      90     100
QTLVNSVNP GKIPKACCVPTLSAISMLYLDENENVVLKNYQDMVVEGCGCR
SI HAI SEQV EP A EQMNSLAI FFNDQDK I RK EE T DA H H
RF T S K DPV V Y N S H RN RS
N S K P E

```

wherein, in each position where more than one amino acid is shown, any one of the amino acids shown may be used. Novel active proteins also are defined by amino acid sequences comprising an active domain beginning at residue number 6 of this sequence, i.e., omitting the N terminal CXXX, or omitting any of the preferred specific combinations such as CKRHP, CRRKQ, CKRHE, etc., resulting in a construct having only six cysteine residues. After this work, PCT 87/01537 was published, and it was observed that the proteins there identified as BMPII a and b and BMPIII each included a region embodying this generic structure. These proteins were not demonstrated to be osteogenic in the published application. However, applicants discovered that a subpart of the amino acid sequence of these proteins, properly folded, and implanted as set forth herein, is active. These are disclosed herein as CBMPIIa, CBMPIIb, and CBMPIII. Also, applicants retrieved a previously unreported gene by probing a human genomic DNA library with COPO. This protein was designated OP1. It comprises a region exhibiting the same generic structure.

[0038] Thus, the preferred osteogenic proteins are expressed from recombinant DNA and comprise amino acid sequences including any of the following sequences:

Vg1 1 10 20 30 40
 CKKRHLVVEFK-DVGWQNWVIAPOGYMANYCYGECFYPLTE
 5 50 60 70
 ILNGSN--H-AILQTLVHSIEPED-IPLPCCVPTKMSP
 80 90 100
 ISMLFYDNNDNVVLRHYENMAVDECGCR

DPP 1 10 20 30 40
 CRRHSLYVDFS-DVGWDDWIVAPLGDAYYCHGKCPFLAD
 15 50 60 70
 HFNSTN--H-AVVQTLVNNNNPGK-VPKACCVPTQLDS
 80 90 100
 VAMLYLNDQSTVVLKNYQEMTVVGCGR

OP1 1 10 20 30 40
 LYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFLNS
 25 50 60 70
 YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
 80 90 100
 ISVLYFDDSSNVILKKYRNMVVRACGCH

OP1 1 10 20 30 40
 CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFLNS
 35 -5
 HQRQA
 50 60 70
 YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
 80 90 100
 ISVLYFDDSSNVILKKYRNMVVRACGCH

CBMP-2a 1 10 20 30 40
 CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECFFPLAD
 50 50 60 70
 HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTLSA
 80 90 100
 ISMLYLDENEKVVLKNYQDMVVEGCGCR

CBMP-2b

1	10	20	30	40
CRRHSLYVDFS-	DVGWNDWIVAPPGYQAFYCHGDCPFPLAD			
	50	60	70	
HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA				
80	90	100		
ISMLYLDEYDKVVLKKNYQEMVVEGCGCR				

CBMP-3

1	10	20	30	40
CARRYLKVDFA-DIGWSEWIISPKSFDAYYCSGACQFPMPK				
	50	60	70	
SLKPSN--H-ATIQSIVRAVGVPPIPEPCCVPEKMSS				
80	90	100		
LSILFFDENKNVVLKVYPNMTVESACR				

COP1

1	10	20	30	40
LYVDFQRDVGWDDWIIAPVDFDAYYCSGACQFPSAD				
	50	60	70	
HFNSTN--H-AVVQTLVNNMNPGRK-VPKPCCVPTELSA				
80	90	100		
ISMLYLDENSTVVLKKNYQEMTVVGCGCR				

COP3

1	10	20	30	40
LYVDFQRDVGWDDWIVAPPGYQAFYCSGACQFPSAD				
	50	60	70	
HFNSTN--H-AVVQTLVNNMNPGRK-VPKPCCVPTELSA				
80	90	100		
ISMLYLDENKVVVLKKNYQEMVVEGCGCR				

COP4

1	10	20	30	40
LYVDFS-DVGWDDWIVAPPGYQAFYCSGACQFPSAD				
	50	60	70	
HFNSTN--H-AVVQTLVNNMNPGRK-VPKPCCVPTELSA				
80	90	100		
ISMLYLDENKVVVLKKNYQEMVVEGCGCR				

Production of Active Proteins

[0042] The following procedure was followed for production of active recombinant proteins. *E. coli* cells containing the fusion proteins were lysed. The fusion proteins were purified by differential solubilization. In the case of the COP1, 3, 4, 5, and 7 fusion proteins, cleavage was with dilute acid, and the resulting cleavage products were passed through a Sephacryl-200HR column. The Sephacryl column separated most of the uncleaved fusion products from the COP1, 3, 4, 5, and 7 analogs. In the case of the COP16 or OP1 fusion protein, cleavage was with a more concentrated acid, and an SP-Trisacryl column was used as an additional purification step. The COP or OP fractions were then subjected to HPLC on a semi-prep C-18 column.

[0043] Initial conditions for refolding of COP analogs or OP1 were at pH 8.0 using Tris, Gu-HCl, dithiothreitol. Final conditions for refolding of COP analogs were at pH 8.0 using Tris, oxidized glutathione, and lower amounts of Gu-HCl and dithiothreitol. Alternatively, the COP or OP1 proteins are suspended in 50 mM HCl, 6 M guanidine-HCl, pH 8.0, for 18 hours at 4°C. Refolding may not be required if the proteins are expressed in animal cells.

Production of Antisera

[0044] Antisera to COP7 and COPS were produced in New Zealand white rabbits. Western blots demonstrate that the antisera react with COP7 and COPS preparations. Antisera to COP7 has been tested for reactivity to naturally sourced bovine osteogenic protein samples. Western blots show a clear reaction with the 30 kD protein and, when reduced, with the 16 kD subunit. The immunoreactive species appears as a closely-spaced doublet in the 16 kD subunit region, similar to the 16 kD doublet seen in Con A blots.

MATRIX PREPARATION

General Consideration of Matrix Properties

[0045] The carrier described in the bioassay section, *infra*, may be replaced by either a biodegradable-synthetic or synthetic-inorganic matrix (e.g., HAP, collagen, tricalcium phosphate, or polylactic acid, polyglycolic acid and various copolymers thereof). Also xenogeneic bone may be used if pretreated as described below.

[0046] Studies have shown that surface charge, particle size, the presence of mineral, and the methodology for combining matrix and osteogenic protein all play a role in achieving successful bone induction. Perturbation of the charge by chemical modification abolishes the inductive response. Particle size influences the quantitative response of new bone; particles between 75 and 420 µm elicit the maximum response. Contamination of the matrix with bone mineral will inhibit bone formation. Most importantly, the procedures used to formulate osteogenic protein onto the matrix are extremely sensitive to the physical and chemical state of both the osteogenic protein and the matrix.

[0047] The sequential cellular reactions at the interface of the bone matrix/OP implants are complex. The multistep cascade includes: binding of fibrin and fibronectin to implanted matrix, chemotaxis of cells, proliferation of fibroblasts, differentiation into chondroblasts, cartilage formation, vascular invasion, bone formation, remodeling, and bone marrow differentiation.

[0048] A successful carrier for osteogenic protein must perform several important functions. It must bind osteogenic protein and act as a slow release delivery system, accommodate each step of the cellular response during bone development, and protect the osteogenic protein from nonspecific proteolysis. In addition, selected materials must be biocompatible *in vivo* and biodegradable; the carrier must act as a temporary scaffold until replaced completely by new bone. Biocompatibility requires that the matrix not induce significant inflammation when implanted and not be rejected by the host animal. Biodegradability requires that the matrix be slowly absorbed by the body of the host during development of new bone or cartilage. Polylactic acid (PLA), polyglycolic acid (PGA), and various combinations have different dissolution rates *in vivo*. In bones, the dissolution rates can vary according to whether the implant is placed in cortical or trabecular bone.

[0049] Matrix geometry, particle size, the presence of surface charge, and porosity or the presence of interstices among the particles of a size sufficient to permit cell infiltration, are all important to successful matrix performance. It is preferred to shape the matrix to the desired form of the new bone and to have dimensions which span non-union defects. Rat studies show that the new bone is formed essentially having the dimensions of the device implanted.

[0050] The matrix may comprise a shape-retaining solid made of loosely adhered particulate material, e.g., with collagen. It may also comprise a molded, porous solid, or simply an aggregation of close-packed particles held in place by surrounding tissue. Masticated muscle or other tissue may also be used. Large allogeneic bone implants can act as a carrier for the matrix if their marrow cavities are cleaned and packed with particles and the dispersed osteogenic protein.

Preparation of Biologically Active Allogenic Matrix

[0051] Demineralized bone matrix is prepared from the dehydrated diaphyseal shafts of rat femur and tibia as described herein to produce a bone particle size which pass through a 420 μm sieve. The bone particles are subjected to dissociative extraction with 4 M guanidine-HCl. Such treatment results in a complete loss of the inherent ability of the bone matrix to induce endochondral bone differentiation. The remaining insoluble material is used to fabricate the matrix. The material is mostly collagenous in nature, and upon implantation, does not induce cartilage and bone. All new preparations are tested for mineral content and false positives before use. The total loss of biological activity of bone matrix is restored when an active osteoinductive protein fraction or a pure protein is reconstituted with the biologically inactive insoluble collagenous matrix. The osteoinductive protein can be obtained from any vertebrate, e.g., bovine, porcine, monkey, or human, or produced using recombinant DNA techniques.

Preparation of Deglycosylated Bone Matrix for Use in Xenogenic Implant

[0052] When osteogenic protein is reconstituted with collagenous bone matrix from other species and implanted in rat, no bone is formed. This suggests that while the osteogenic protein is xenogenic (not species specific), while the matrix is species specific and cannot be implanted cross species perhaps due to intrinsic immunogenic or inhibitory components. Thus, heretofore, for bone-based matrices, in order for the osteogenic protein to exhibit its full bone inducing activity, a species specific collagenous bone matrix was required.

[0053] The major component of all bone matrices is Type I collagen. In addition to collagen, extracted bone includes non-collagenous proteins which may account for 5% of its mass. Many non-collagenous components of bone matrix are glycoproteins. Although the biological significance of the glycoproteins in bone formation is not known, they may present themselves as potent antigens by virtue of their carbohydrate content and may constitute immunogenic and/or inhibitory components that are present in xenogenic matrix.

[0054] It has now been discovered that a collagenous bone matrix may be used as a carrier to effect bone inducing activity in xenogenic implants, if one first removes the immunogenic and inhibitory components from the matrix. The matrix is deglycosylated chemically using, for example, hydrogen fluoride to achieve this purpose.

[0055] Bovine bone residue prepared as described above is sieved, and particles of the 74-420 μm are collected. The sample is dried in vacuo over P_2O_5 , transferred to the reaction vessel and anhydrous hydrogen fluoride (HF) (10-20 ml/g of matrix) is then distilled onto the sample at -70°C . The vessel is allowed to warm to 0°C . and the reaction mixture is stirred at this temperature for 120 min. After evaporation of the HF in vacuo, the residue is dried thoroughly in vacuo over KOH pellets to remove any remaining traces of acid.

[0056] Extent of deglycosylation can be determined from carbohydrate analysis of matrix samples taken before and after treatment with HF, after washing the samples appropriately to remove non-covalently bound carbohydrates.

[0057] The deglycosylated bone matrix is next treated as set forth below:

1) suspend in TBS (Tris-buffered Saline) 1g/200 ml and stir at 4°C for 2 hrs; or in 6 M urea, 50 mM Tris-HCl, 500 mM NaCl, pH 7.0 (UTBS) and stir at RT for 30 min;

2) centrifuge and wash with TBS or UTBS as in step 1; and

3) centrifuge; discard supernatant; water wash residue; and then lyophilize.

FABRICATION OF DEVICE

[0058] Fabrication of osteogenic devices using any of the matrices set forth above with any of the osteogenic proteins described above may be performed as follows.

A. Ethanol precipitation

[0059] In this procedure, matrix is added to osteogenic protein in guanidine-HCl. Samples are vortexed and incubated at a low temperature. Samples are then further vortexed. Cold absolute ethanol is added to the mixture which is then stirred and incubated. After centrifugation (microfuge high speed) the supernatant is discarded. The reconstituted matrix is washed with cold concentrated ethanol in water and then lyophilized.

B. Acetonitrile Trifluoroacetic Acid Lyophilization

[0060] In this procedure, osteogenic protein in an acetonitrile trifluoroacetic acid (ACN/TFA) solution is added to the

carrier. Samples are vigorously vortexed many times and then lyophilized.

C. Urea Lyophilization

- 5 **[0061]** For those proteins that are prepared in urea buffer, the protein is mixed with the matrix, vortexed many times, and then lyophilized. The lyophilized material may be used "as is" for implants.

IN VIVO RAT BIOASSAY

- 10 **[0062]** Several of the synthetic proteins have been incorporated in matrices to produce osteogenic devices, and assayed in rat for endochondral bone. Studies in rats show the osteogenic effect to be dependent on the dose of osteogenic protein dispersed in the osteogenic device. No activity is observed if the matrix is implanted alone. The following sets forth guidelines for how the osteogenic devices disclosed herein can be assayed for evaluating protein constructs and matrices for biological activity.

A. Subcutaneous Implantation

- 15 **[0063]** The bioassay for bone induction as described by Sampath and Reddi (Proc. Natl. Acad. Sci. USA (1983) 80: 6591-6595), herein incorporated by reference, is used to assess endochondral bone differentiation activity. This assay consists of implanting the test samples in subcutaneous sites in allogenic recipient rats under ether anesthesia. Male Long-Evans rats, aged 28-32 days, were used. A vertical incision (1 cm) is made under sterile conditions in the skin over the thoracic region, and a pocket is prepared by blunt dissection. Approximately 25 mg of the test sample is implanted deep into the pocket and the incision is closed with a metallic skin clip. The day of implantation is designated as day one of the experiment. Implants were removed on day 12. The heterotopic site allows for the study of bone induction without the possible ambiguities resulting from the use of orthotopic sites.

B. Cellular Events

- 20 **[0064]** The implant model in rats exhibits a controlled progression through the stages of matrix induced endochondral bone development including: (1) transient infiltration by polymorphonuclear leukocytes on day one; (2) mesenchymal cell migration and proliferation on days two and three; (3) chondrocyte appearance on days five and six; (4) cartilage matrix formation on day seven; (5) cartilage calcification on day eight; (6) vascular invasion, appearance of osteoblasts, and formation of new bone on days nine and ten; (7) appearance of osteoblastic and bone remodeling and dissolution of the implanted matrix on days twelve to eighteen; and (8) hematopoietic bone marrow differentiation in the ossicle on day twenty-one. The results show that the shape of the new bone conforms to the shape of the implanted matrix.

C. Histological Evaluation

- 25 **[0065]** Histological sectioning and staining is preferred to determine the extent of osteogenesis in the implants. Implants are fixed in Bouins Solution, embedded in parafilm, cut into 6-8 mm sections. Staining with toluidine blue or hemotoxylin/eosin demonstrates clearly the ultimate development of endochondral bone. Twelve day implants are usually sufficient to determine whether the implants show bone inducing activity.

D. Biological Markers

- 30 **[0066]** Alkaline phosphatase activity may be used as a marker for osteogenesis. The enzyme activity may be determined Spectrophotometrically after homogenization of the implant. The activity peaks at 9-10 days in vivo and thereafter slowly declines. Implants showing no bone development by histology should have little or no alkaline phosphatase activity under these assay conditions. The assay is useful for quantitation and obtaining an estimate of bone formation very quickly after the implants are removed from the rat. Alternatively the amount of bone formation can be determined by measuring the calcium content of the implant.

[0067] The osteogenic activity due to osteogenic protein is represented by "bone forming units". One bone forming unit represents the amount of protein that is needed for half maximal bone forming activity as compared to rat demineralized bone matrix as control and determined by calcium content of the implant on day 12.

- 35 **[0068]** Devices that contained only rat carrier show complete absence of new bone formation. The implant consists of carrier rat matrix and surrounding mesenchymal cells. Again, the devices that contained rat carrier and not correctly folded (or biologically inactive) recombinant protein also showed complete absence of bone formation. These implants are scored as cartilage formation (-) and bone formation (-). The endochondral bone formation activity is scored as

zero percent (0%) (FIGURE 3A).

[0069] Implants included biologically active recombinant protein, however, showed evidence of endochondral bone formation. Histologically they showed new cartilage and bone formation.

[0070] The cartilage formation is scored as (+) by the presence of metachromatically stained chondrocytes in the center of the implant, as (++) by the presence of numerous chondrocytes in many areas of the implant and as (+++) by the presence of abundant chondrocytes forming cartilage matrix and the appearance of hypertrophied chondrocytes accompanying cartilage calcification (FIGURE 3B).

[0071] The bone formation is scored as (+) by the presence of osteoblast surrounding vascular endothelium forming new matrix, as (++) by the formation of bone due to osteoblasts (as indicated by arrows) and further bone remodeling by the appearance of osteoclasts in opposition to the newly formed bone matrix. Vascular invasion is evident in these implants (FIGURE 3B). Formation is scored as (+++) by the presence of extensive remodeled bone which results in the formation of ossicles.

[0072] The overall bone inducing activity due to recombinant protein is represented as percent response of endochondral bone formation (see TABLE 2 below).

TABLE 2

HISTOLOGICAL EVALUATION OF RECOMBINANT BONE INDUCTIVE PROTEINS			
Sample No.	Implanted Protein	Cartilage Formation	Bone Formation
260-54	COP-5	+++	++
279-5	COP-5	++	+
285-13	COP-5	+++	++
277-7	COP-7	+++	++
277-8	COP-7	+++	++
277-9	COP-7	++	+
285-14	COP-7	+++	++
285-24	COP-7	++	+
285-25	COP-7	++	++
314-6	COP-16	+++	+++
314-15	COP-16	++	+
314-16	COP-16	++	+
314-12	OP-1	++	+

[0073] The invention relates to an osteogenic device for implantation in a mammal, said device comprising a biocompatible, in vivo biodegradable matrix defining pores of a dimension sufficient to permit influx, proliferation and differentiation of migratory progenitor cells from the body of said mammal; and a protein, produced by expression of recombinant DNA in a host cell, comprising one or more polypeptide chains, each of which comprises an amino acid sequence sufficiently duplicative of the sequence of COP5, COP7, COP16, or OP1 such that said protein is capable of inducing endochondral bone formation in association with said matrix when implanted in a mammal.

[0074] Also covered by the invention is a device for implantation in a mammal, said device comprising a biocompatible, in vivo biodegradable matrix defining pores of a dimension sufficient to permit influx, proliferation and differentiation of migratory progenitor cells from the body of said mammal; and a protein, produced by expression of recombinant DNA in a host cell, comprising one or more polypeptide chains, each of which has less than about 200 amino acids, and each of which comprises a sequence sufficiently duplicative of the sequence of COP5, COP7, COP16, or OP1 such that said protein is capable of inducing cartilage formation in association with said matrix when implanted in a mammal.

[0075] The sequence may comprise

```

      10      20      30      40      50
CXXXLXVXFDCXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXNHAXX
      60      70      80      90     100
QXXVXXXNXXXXPXXCCPXXXXXXXXXXVXLXXYXXMXVXXCXCX

```

wherein each X independently represents an amino acid; or

```

      10      20      30      40      50
5      LXVFXDXGWXXWXXXPXGXAXYCXCXCXPPXXXXXXXXNHAXX
      60      70      80      90     100
      QXXVXXNXNXXXPXXCCXPXXXXXXXXLXXXXXXXXVXLXXYXXMXVXXCXCX

```

10 wherein each X independently represents an amino acid; or

```

      10      20      30      40      50
15     CKRHPLYVDFRDVGWNDWIVAPPGYHAFYCHGECFPFLADHLNSTNHAIV
      RKRS K S S L QE VIS E FD Y E A AY MPESMKAS VI
      KE F E K I DN L N S Q ITK F P TL
      Q A S K
      60      70      80      90     100
20     QTLVNSVNP GKIPKACCVPTELSAISMLYLDENENVVLKKNYQDMVVEGCGCR
      SI HAI SEQV EP A EQMNSLAI FFNDQDK I RK EE T DA H H
      RF T S K DPV V Y N S H RN RS
      N S K P E

```

25 wherein, in each position where more than one amino acid is shown, any one of the amino acids shown may be in that position; or

```

      10      20      30      40      50
30     LYVDFRDVGWNDWIVAPPGYHAFYCHGECFPFLADHLNSTNHAIV
      K S S L QE VIS E FD Y E A AY MPESMKAS VI
      F E K I DN L N S Q ITK F P TL
      A S K
      60      70      80      90     100
35     QTLVNSVNP GKIPKACCVPTELSAISMLYLDENENVVLKKNYQDMVVEGCGCR
      SI HAI SEQV EP A EQMNSLAI FFNDQDK I RK EE T DA H H
      RF T S K DPV V Y N S H RN RS
      N S K P E
40

```

45 wherein, in each position where more than one amino acid is shown, any one of the amino acids shown may be in that position; or

```

      1      10      20      30      40
50     Vg1 CKKRHLYVEFK-DVGWQNWVIAPQGYMANYCYGECPYPLTE
      50      60      70
      ILNGSN--H-AILQTLVHSIEPED-IPLPCCVPTKMSP
      80      90     100
      ISMLFYDNNNDNVVLRHYENMAVDECGCR

```

or

5 DPP 1 10 20 30 40
 CRRHSLYVDFS-DVGWDDWIVAPLGDAYYCHGKCPFPLAD
 50 60 70
 HFNSTN--H-AVVQTLVNNNNPGK-VPKACCVPTQLDS
 80 90 100
 10 VAMLYLNDQSTVVLKQEMTVVGCGR

or

15 OP1 1 10 20 30 40
 LYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPPLNS
 50 60 70
 20 YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
 80 90 100
 ISVLYFDDSSNVILKKYRNMVVRACGCH

25 or

30 OP1 1 10 20 30 40
 CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPPLNS
 50 60 70
 YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
 80 90 100
 35 ISVLYFDDSSNVILKKYRNMVVRACGCH

or

40 CBMP-2a 1 10 20 30 40
 CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPPFLAD
 50 60 70
 45 HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
 80 90 100
 ISMLYLDENEKVVLKQDMVVEGCGCR

or

50 CBMP-2b 1 10 20 30 40
 CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
 50 60 70
 55 HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
 80 90 100
 ISMLYLDEYDKVVLKQEMVVEGCGCR

or

5 CBMP-3 1 10 20 30 40
 CARRYLKVDFA-DIGWSEWIIISPKSFDAYYCSGACQFMPK
 50 60 70
 SLKPSN--H-ATIQSIVRAVGVPGIPEPCCVPEKMSS
 80 90 100
 LSILFFDENKNVVLKVYPNMTVESACR

10

or

15 COP1 1 10 20 30 40
 LYVDFQRDVGWDDWIIAPVDFDAYYCSGACQFPSAD
 50 60 70
 HFNSTN--H-AVVQTLVNNMNPBK-VPKPCCVPTLSA
 80 90 100
 ISMLYLDENSTVVLKKNYQEMTVVGCGR

20

or

25 COP3 1 10 20 30 40
 LYVDFQRDVGWDDWIVAPPGYQAFYCSGACQFPSAD
 50 60 70
 HFNSTN--H-AVVQTLVNNMNPBK-VPKPCCVPTLSA
 80 90 100
 ISMLYLDENEKVVVLKKNYQEMVVEGCGR

30

35 or

40 COP4 1 10 20 30 40
 LYVDFS-DVGWDDWIVAPPGYQAFYCSGACQFPSAD
 50 60 70
 HFNSTN--H-AVVQTLVNNMNPBK-VPKPCCVPTLSA
 80 90 100
 ISMLYLDENEKVVVLKKNYQEMVVEGCGR

40

45 or

50 COP5 1 10 20 30 40
 LYVDFS-DVGWDDWIVAPPGYQAFYCHGECPFPLAD
 50 60 70
 HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTLSA
 80 90 100
 ISMLYLDENEKVVVLKKNYQEMVVEGCGR

50

55

or

or

10
PKHHSQRARKKNKN
1 10 20 30 40
COP16 CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGECPPFLAD
50 60 70
HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTLSA
80 90 100
ISMLYLDENEKVVLKNYOEMVVEGCGCR

[0077] The invention also relates to an osteogenic protein, produced by expression of recombinant DNA in a host cell, comprising one or more polypeptide chains each of which comprises a sequence sufficiently duplicative of the sequence of COP5, COP7, COP16, or OP1 such that said protein is capable of inducing endochondral bone formation in association with a matrix when implanted in a mammal.

[0078] In another embodiment, the invention relates to a protein, produced by expression of recombinant DNA in a host cell, comprising one or more polypeptide chains less than about 200 amino acids long and comprising a sequence sufficiently duplicative of the sequence of COP5, COP7, COP16, or OP1 such that said protein is capable of inducing cartilage formation in association with a matrix when implanted in a mammal.

[0079] The protein may be unglycosylated, and may comprise the amino acid sequences:

10 20 30 40 50
 CXXXLXVFXDXGWXXWXXXPXGXAXYCXXGXCPXXXXXXXNHAXX
 60 70 80 90 100
 OXXVXXXNXXXXPXXCCPXXXXXXXXLXXXXXXXXVXLXXYXXMXVXXCXCX

or

10 20 30 40 50
 LXVFXDXGWXXWXXXPXGXAXYCXGXCXPPXXXXXXXXNHAXX
 60 70 80 90 100
 OXXVXXNXXXPPXXCCPXXXXXXXXLXXXXXXXXVXLXXYXXMXVXXCXCX

wherein each X independently represents an amino acid.

[0080] The protein of the invention may comprise the amino acid sequences:

10 20 30 40 50
 CKRHPLYVDFRDVGWNDWIVAPPGYHAFYCHGECPPFLADHLNSTNHAIV
 5 RKRS K S S L QE VIS E FD Y E A AY MPESMKAS VI
 KE F E K I DN L N S Q ITK F P TL
 Q A S K
 60 70 80 90 100
 QTLVNSVNPFGKIPKACCVPTELSAISMLYLDENENVVLKKNYQDMVVEGCGCR
 10 SI HAI SEQV EP A EQMNSLAI FFNDQDK I RK EE T DA H H
 RF T S K DPV V Y N S H RN RS
 N S K P E

15 wherein, in each position where more than one amino acid is shown, any one of the amino acids shown may be in that position.

[0081] The protein of the invention may comprise the amino acid sequences:

20 10 20 30 40 50
 LYVDFRDVGWNDWIVAPPGYHAFYCHGECPPFLADHLNSTNHAIV
 K S S L QE VIS E FD Y E A AY MPESMKAS VI
 F E K I DN L N S Q ITK F P TL
 A S K
 25 60 70 80 90 100
 QTLVNSVNPFGKIPKACCVPTELSAISMLYLDENENVVLKKNYQDMVVEGCGCR
 SI HAI SEQV EP A EQMNSLAI FFNDQDK I RK EE T DA H H
 RF T S K DPV V Y N S H RN RS
 N S K P E
 30

wherein, in each position where more than one amino acid is shown, any one of the amino acids shown may be in that position.

[0082] The protein of the invention may comprise the amino acid sequences:

40 Vg1 1 10 20 30 40
 CKKRHLYVEFK-DVGWQNWVIA PQGYMAN YCYGEC PYPLTE
 50 60 70
 ILNGSN--H-AILQTLVHSIEPED-IPLPCCVPTKMSP
 80 90 100
 ISMLFYDNNDNVVLRHYENMAVDECGCR

or

50 DPP 1 10 20 30 40
 CRRHSLYVDFS-DVGWDDWIVAPLGYDAYYCHGKCPFLAD
 50 60 70
 HFNSTN--H-AVVQTLVNNNNPGK-VPKACCVPTQLDS
 80 90 100
 VAMLYLNDQSTVVLKKNYQEMTVVVGCGCR
 55

or

1 10 20 30 40
 OP1 LYVSFR-DLGWQDWIIAPEGYAAYYCEGECAPFLNS

5 50 60 70
 YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
 80 90 100
 ISVLYFDDSSNVILKKYRNMVVRACGCH

or

15 -5
 HQRQA
 1 10 20 30 40
 OP1 CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAPFLNS
 50 60 70
 YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
 20 80 90 100
 ISVLYFDDSSNVILKKYRNMVVRACGCH

or

25
 1 10 20 30 40
 CBMP-2a CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
 50 60 70
 HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTLSA
 80 90 100
 ISMLYLDENEKVVLKNYQDMVVEGCGCR

or

40
 1 10 20 30 40
 CBMP-2b CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
 50 60 70
 HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTLSA
 45 80 90 100
 ISMLYLDYDKVVLKNYQEMVVEGCGCR

or

CBMP-3 1 10 20 30 40
 CARRYLKVDFA-DIGWSEWIIISPKSFDAYYCSGACQFPMPK
 50 60 70
 SLKPSN--H-ATIQSIVRAVGVPGIPEPCCVPEKMSS
 80 90 100
 LSILFFDENKNVVLKVYPNMTVESACR

or

COP1 1 10 20 30 40
 LYVDFQRDVGWDDWIIAPVDFDAYYCSGACQFPSAD
 50 60 70
 HFNSTN--H-AVVQTLVNNMNPBK-VPKPCCVPTLSA
 80 90 100
 ISMLYLDENSTVVLKNYQEMTVVGCGR

or

COP3 1 10 20 30 40
 LYVDFQRDVGWDDWIVAPPGYQAFYCSGACQFPSAD
 50 60 70
 HFNSTN--H-AVVQTLVNNMNPBK-VPKPCCVPTLSA
 80 90 100
 ISMLYLDENEKVVVLKNYQEMVVEGCGCR

or

COP4 1 10 20 30 40
 LYVDFS-DVGWDDWIVAPPGYQAFYCSGACQFPSAD
 50 60 70
 HFNSTN--H-AVVQTLVNNMNPBK-VPKPCCVPTLSA
 80 90 100
 ISMLYLDENEKVVVLKNYQEMVVEGCGCR

or

2. The protein of claim 1 wherein the protein comprises a region containing seven cysteine residues positioned in the relative positions shown in the sequence:

5 CXXXXLXVFXDXGWXXWXXXPXGXXAXYCXXGXCXXPXXXXXXXXXNHAXX
QXXVXXXNXXXXPXXCCXPXXXXXXXXXXLXXXXXXXXVXLXXYXXMXVXXCXCX.

- 10 3. The protein of claim 1 or claim 2 which is a recombinant protein produced by expression in a host cell.
4. A process for producing active synthetic osteogenic protein as defined in claim 1, said protein comprising a pair of polypeptide chains disulphide bonded to produce a dimeric species, the process comprising the steps of:

15 (a) producing a consensus amino acid sequence by comparing amino acid sequences of proteins of the TGF-beta gene family, which consensus sequence preserves the disulphide crosslinking pattern present in the sequence:

20 (i) LXVFXDXGWXXWXXXPXGXXAXYCXXGXCXXPXXXXXXXXX
HAXXQXXVXXXNXXXXPXXCCXPXXXXXXXXXXLXXXXXXXXVXL
XXYXXMXVXXCXCX;

25 or

30 (ii) CXXXXLXVFXDXGWXXWXXXPXGXXAXYCXXGXCXXPXXXX
XXXXNHAXXQXXVXXXNXXXXPXXCCXPXXXXXXXXXXLXXXX
XXVXLXXYXXMXVXXCXCX;

35 wherein the letters indicate the amino acid residues of standard single letter code and each X represents any amino acid;

(b) constructing a synthetic gene encoding the consensus amino acid sequence of step (a), for example by assembling chemically synthesised oligonucleotides;

40 (c) introducing the synthetic gene of step (b) into a prokaryotic or eukaryotic host cell;

(d) expressing the synthetic gene to produce a putative synthetic osteogenic protein;

45 (e) assaying the putative synthetic osteogenic protein to determine whether it is active in inducing endochondral bone formation when implanted in a mammal, and optionally

(f) repeating steps (a) to (e) (or mutagenizing the synthetic gene and repeating steps (c) to (e) if the putative synthetic osteogenic protein is inactive when so assayed.

- 50 5. The process of claim 4 wherein the consensus nucleic acid sequence comprises the sequence:

55 (a) LXVFXDXGWXXWXXXPXGXXAXYCXXGXCXXPXXXXXXXXX
HAXXQXXVXXXNXXXXPXXCCXPXXXXXXXXXXLXXXXXXXXVXL
XXYXXMXVXXCXCX;

or

(b) CXXXXLXVFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXX
 XXXXNHAXXQXXVXXXNXXXPXXCCPXXXXXXXXXXLXXXXX
 XXVXLXXYXXMXVXXCXCX;

wherein the letters indicated the amino acid residues of standard single letter code and each X represents any amino acid.

6. The process of any one of claims 4-5 wherein the synthetic gene of step (b) comprises a nucleotide sequence which encodes a synthetic polypeptide chain having the same number of cysteine residues in the same relative positions as COPS, COP7, COP16 or OP1.
7. The process of any one of claims 4-6 wherein the synthetic gene of step (b) comprises a nucleotide sequence which encodes a synthetic polypeptide chain sharing one or more amino acids at corresponding positions in any of the sequences: Vg1, DPP, OP1, CBMP-2a, CBMP-2b, CBMP-3, COP1, COP3, COP4, COPS, COP7 or COP16.
8. The process of any one of claims 4-7 wherein the synthetic gene of step (b) comprises a nucleotide sequence which encodes a synthetic polypeptide chain comprising the amino acid sequence:

(a)

	10	20	30	40	50
	CKRHPLYVDFRDVGWNDWIVPPGYHAFYCHGECPPFLADHLNSTNHAIV				
	RKRS	K S S L	QE VIS E	FD Y E A AY	MPESMKAS VI
	KE F E	K I DN	L N S	Q ITK F P	TL
	Q	A S	K		
	60	70	80	90	100
	QTLVNSVNP GKIPKACCVPTELSAISMLYLDENENVVLKKNYQDMVVEGCGCR				
	SI HAI	SEQV EP	A EQMNSLAI	FFNDQDK I RK	EE T DA H H
	RF	T S	K DPV V	Y N S	H RN RS
	N	S		K	P E

wherein, in each position where more than one amino acid is shown, any one of the amino acids shown may be in that position; or

(b)

	10	20	30	40	50
	LYVDFRDVGWNDWIVAPPGYHAFYCHGECPPFLADHLNSTNHAIV				
	K S S L	QE VIS E	FD Y E A AY	MPESMKAS	VI
	F E K I	DN	L N S	Q ITK F P	TL
	A	S	K		
	60	70	80	90	100
	QTLVNSVNP GKIPKACCVPTELSAISMLYLDENENVVLKKNYQDMVVEGCGCR				
	SI HAI	SEQV EP	A EQMNSLAI	FFNDQDK I RK	EE T DA H H
	RF	T S	X DPV V	Y N S	H RN RS
	N	S		K	P E

1 10 20 30 40
CRRHSLYVDFS-DVGWDDWIVAPLGYDAYYCHGKCPFFPLAD
 50 60 70
HFNSTN--H-AVVQTLVNNNNPGK-VPKACCVPTQLDS
 80 90 100
VAMLYLNDQSTVVLKNYQEMTVVGCGR; or

1 10 20 30 40
LYVSFR-DLGWQDWIIAPEGYAAYYCEGECAPFLNS
 50 60 70
YMNATN--H-AIVQTLVHFIFNPET-VPKPCCAPTQLNA
 80 90 100
ISVLYFDDSSNVILKKYRNMVVRACGCH; or

```

-5
HQRQA
1      10      20      30      40
CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAPFLNS
      50      60      70
YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
      80      90     100
ISVLYFDDSSNVILKKYRNMVVVRACGCH; or

```

(f)

5 1 10 20 30 40
 LYVDFQRDVGWDDWIIAPVDFDAYYCSGACQFPSAD
 50 60 70
 10 HFNSTN--H-AVVQTLVNNMNPBK-VPKPCCVPTELSA
 80 90 100
 ISMLYLDENSTVVLKNYQEMTVVGCGR; or

15

(g)

20 1 10 20 30 40
 LYVDFQRDVGWDDWIVAPPGYQAFYCSGACQFPSAD
 50 60 70
 25 HFNSTN--H-AVVQTLVNNMNPBK-VPKPCCVPTELSA
 80 90 100
 30 ISMLYLDENEKVVVLKNYQEMVVEGCGCR; or

35

(h)

40 1 10 20 30 40
 LYVDFS-DVGWDDWIVAPPGYQAFYCSGACQFPSAD
 50 60 70
 HFNSTN--H-AVVQTLVNNMNPBK-VPKPCCVPTELSA
 45 80 90 100
 ISMLYLDENEKVVVLKKYQEMVVEGCGCR.

- 50 9. The protein of any one of claims 1-3 wherein the protein comprises a synthetic polypeptide chain of less than 200 amino acids.
10. The process of any one of claims 4-8 wherein the protein comprises a synthetic polypeptide chain of less than 200 amino acids.
- 55 11. The protein of claim 3 wherein the host cell is a prokaryotic or eukaryotic host cell, for example E. coli, bacillus, yeasts or animal cells (e.g. CHO or myeloma).
12. The process of any one of claims 4-8 or 10 wherein the host cell is a prokaryotic or eukaryotic host cell, for example

E. coli, bacillus, yeasts or animal cells (e.g. CHO or myeloma).

13. The protein of any one of claims 1-3 wherein the protein is unglycosylated.

14. The process of any one of claims 4-8 or 10 wherein the protein is unglycosylated.

15. A composition comprising protein comprising a pair of polypeptide chains disulphide bonded to produce a dimeric species, the protein being as defined in claim 1, 2 or 3.

16. An osteogenic device comprising a matrix in which is disposed the protein of claim 1, 2 or 3.

17. An osteogenic device for implantation in a mammal, the device comprising: (a) a biocompatible, in vivo biodegradable matrix defining a scaffold of dimensions sufficient to permit the attachment, proliferation and differentiation of migratory progenitor cells from the body of said mammal, and (b) the protein of claim 1, 2 or 3 disposed in said matrix and accessible to said cells.

18. The device of claim 16 or claim 17 wherein said matrix comprises close-packed particulate matter having a particle size within the range of 70 to 850 μm (e.g. 70 to 420 μm).

19. The device of any one of claims 16-18 wherein said matrix comprises: (a) allogenic bone, e.g. demineralized, protein extracted, particulate, allogenic bone, (b) demineralized, protein extracted, particulate, deglycosylated xenogenic bone, (c) demineralized, protein extracted, particulate xenogenic bone treated with HF or a protease, (d) materials selected from collagen, hydroxyapatite, calcium phosphates (e.g. tricalcium phosphate) and polymers comprising glycolic acid and/or lactic acid monomers, (e) a shape-retaining solid of loosely adhered particulate material e.g. collagen, (f) a porous solid or (g) masticated tissue, e.g. muscle.

20. The device of any one of claims 16 to 19 disposed within the marrow cavity of allogenic bone.

21. The device of any of claims 16 to 20 for use in therapy, e.g. for inducing local cartilage and/or endochondral or heterotopic bone formation in a mammal by implanting the device in a mammal at a locus accessible to migratory progenitor cells.

22. The device of claim 21 for the formation of shaped heterotopic bone, wherein the shape of the heterotopic bone formed conforms to that of the implanted device.

23. Use of the protein of claim 1, 2 or 3 for the manufacture of: (a) a device according to any of claims 16 to 21, or (b) a medicament, the medicament or device being for inducing local cartilage and/or endochondral or heterotopic bone formation in a mammal by introducing the medicament or device in a mammal at a locus accessible to migratory progenitor cells for periodontal treatment, cartilage repair and for the treatment of osteoarthritis or to correct non-union fractures, acquired or congenital craniofacial and other skeletal or dental anomalies.

24. The use of claim 23 for the formation of shaped heterotopic bone, wherein the shape of the heterotopic bone formed conforms to that of the implanted device.

25. A synthetic nucleic acid molecule for use in the process of any one of claims 4 to 8.

26. A host cell for use in the process of any one of claims 4 to 8 which contains the synthetic nucleic acid molecule of claim 25.

27. The host cell of claim 26 which is a prokaryotic or eukaryotic host cell, for example E. coli, bacillus, yeasts or animal cells (e.g. CHO or myeloma).

28. An isolated DNA sequence which encodes the amino acid sequence of the protein as defined in any one of claims 1-3 or 9.

Patentansprüche

1. Osteogenes Protein, das zwei Polypeptidketten umfasst, die im nicht-reduzierten Zustand gebunden sind, so dass sie eine homo- oder heterodimere Spezies mit einer solchen Konformation bilden, dass die zwei Polypeptidketten dazu in der Lage sind, eine Ersatzknochenbildung zu induzieren, wenn sie in einer Matrix angeordnet und einem Säugetier implantiert werden, wobei das Protein einen Bereich umfasst, der sechs Cystein-Reste enthält, die in den in der Sequenz dargestellten relativen Positionen angeordnet sind:

LXVFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXXNHAXXQXXVX
XXNXXXXPXXCCXPXXXXXXXXXXLXXXXXXXXVXLXXYXXMXVXXCXCX;

wobei die Buchstaben die Aminosäurereste im Standard Ein-Buchstaben-Code angeben und jedes X irgendeine Aminosäure repräsentiert, unter dem Vorbehalt, dass dpp ausgenommen ist.

2. Protein nach Anspruch 1,
wobei das Protein einen Bereich umfasst, der sieben Cystein-Reste enthält, die in den in der Sequenz dargestellten relativen Positionen angeordnet sind:

CXXXXLXVFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXXNHAXX
QXXVXXXNXXXXPXXCCXPXXXXXXXXXXLXXXXXXXXVXLXXYXXMXVXXCXCX.

3. Protein nach Anspruch 1 oder Anspruch 2,
das ein rekombinantes Protein ist, das durch Expression in einer Wirtszelle hergestellt wird.
4. Verfahren zur Herstellung eines aktiven synthetischen osteogenen Proteins, wie in Anspruch 1 definiert, wobei das Protein zwei Polypeptidketten umfasst, die durch DisulfidBrücken verbunden sind, so dass eine dimere Spezies erzeugt wird, wobei das Verfahren die folgenden Schritte umfasst:

(a) Erzeugen einer Aminosäure-Consensussequenz durch Vergleich von Aminosäuresequenzen von Proteinen der TGF-beta-Genfamilie, wobei die Consensussequenz das Disulfidvernetzungsmuster erhält, das in der folgenden Sequenz vorliegt:

(i) LXVFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXXN
HAXXQXXVXXXNXXXXPXXCCXPXXXXXXXXXXLXXXXXXXXVXL
XXYXXMXVXXCXCX;

oder

(ii) CXXXXLXVFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXX
XXXXNHAXXQXXVXXXNXXXXPXXCCXPXXXXXXXXXXLXXXX
XXVXLXXYXXMXVXXCXCX;

wobei die Buchstaben die Aminosäurereste im Standard Ein-Buchstaben-Code anzeigen und jedes X irgendeine Aminosäure repräsentiert;

(b) Konstruieren eines synthetischen Gens, das die Aminosäure-Consensussequenz von Schritt (a) kodiert,

beispielsweise durch Zusammenfügen von chemisch synthetisierten Oligonukleotiden;

(c) Einbringen des synthetischen Gens aus Schritt (b) in eine prokaryontische oder eukaryontische Wirtszelle;

(d) Exprimieren des synthetischen Gens zur Erzeugung eines putativen synthetischen osteogenen Proteins;

(e) Untersuchen des putativen synthetischen osteogenen Proteins, um zu bestimmen, ob es zur Induktion einer Ersatzknochenbildung wirksam ist, wenn es einem Säugetier implantiert wird, und wahlweise

(f) Wiederholen der Schritte (a) bis (e) (oder Mutagenisieren des synthetischen Gens und Wiederholen der Schritte (c) bis (e), wenn das putative synthetische osteogene Protein inaktiv ist, wenn es so geprüft wird).

5. Verfahren nach Anspruch 4,

wobei die Nukleinsäure-Consensussequenz die folgende Sequenz umfasst:

(a) LXVXF~~DX~~GWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXN
 HAXXQXXVXXNXXXPXXCCXPXXXXXXXXLXXXXXXXXVXL
 XXYXXMXVXXCXCX;

oder

(b) CXXXXLXVXF~~DX~~GWXXWXXXPXGXXAXYCXGXCXXPXXXX
 XXXXNHAXXQXXVXXNXXXPXXCCXPXXXXXXXXLXXXX
 XXVXLXXYXXMXVXXCXCX;

wobei die Buchstaben die Aminosäurereste im Standard Ein-Buchstaben-Code angeben und jedes X irgendeine Aminosäure repräsentiert.

6. Verfahren nach einem oder mehreren der Ansprüche 4 bis 5,

wobei das synthetische Gen aus Schritt (b) eine Nukleotidsequenz umfasst, die eine synthetische Polypeptidkette mit derselben Anzahl an Cystein-Resten in denselben relativen Positionen kodiert, wie COP5, COP7, COP16 oder OP1.

7. Verfahren nach irgendeinem der Ansprüche 4 bis 6,

wobei das synthetische Gen aus Schritt (b) eine Nukleotidsequenz umfasst, die eine synthetische Polypeptidkette kodiert, die eine oder mehrere Aminosäuren an den entsprechenden Positionen in irgendeiner der Sequenzen: Vg1, DPP, OP1, CBMP-2a, CMBP-2b, CBMP-3, COP1, COP3, COP4, COP5, COP7 oder COP16, umfasst.

8. Verfahren nach einem der Ansprüche 4 bis 7,

wobei das synthetische Gen aus Schritt (b) eine Nukleotidsequenz umfasst, die eine synthetische Polypeptidkette kodiert, die die folgende Aminosäure umfasst:

(a)

	10	20	30	40	50
5	CKRHPLYVDFRDVGWNDWIVPPGYHAFYCHGECPPFLADHLNSTNHAIV				
	RKRS	K S S L	QE VIS E	FD Y E A	AY MPESMKAS VI
	KE F	E K I	DN L	N S Q	ITK F P TL
	Q	A S	K		
	60	70	80	90	100
10	QTLVNSVNPNGKIPKACCVPTELSAISMLYLDENENVVLKKNYQDMVVEGCGCR				
	SI HAI	SEQV EP	A EQMNSLAI	FFNDQDK I	RK EE T DA H H
	RF	T S	K DPV V	Y N S	H RN RS
	N	S		K	P E

wobei in jeder Position, in der mehr als eine Aminosäure dargestellt ist, irgendeine der dargestellten Aminosäuren in dieser Position vorliegen kann; oder

(b)

	10	20	30	40	50
25	LYVDFRDVGWNDWIVAPPGYHAFYCHGECPPFLADHLNSTNHAIV				
	K S S L	QE VIS E	FD Y E A	AY MPESMKAS	VI
	F E K I	DN L	N S Q	ITK F P	TL
	A	S	K		
	60	70	80	90	100
30	QTLVNSVNPNGKIPKACCVPTELSAISMLYLDENENVVLKKNYQDMVVEGCGCR				
	SI HAI	SEQV EP	A EQMNSLAI	FFNDQDK I	RK EE T DA H H
	RF	T S	X DPV V	Y N S	H RN RS
	N	S		K	P E

wobei in jeder Position, in der mehr als eine Aminosäure dargestellt ist, irgendeine der dargestellten Aminosäuren in dieser Position vorliegen kann; oder

(c)

	1	10	20	30	40
45	CRRHSLYVDFS-DVGWDDWIVAPLGDAYYCHGKCPFLAD				
		50	60	70	
	HFNSTN--H-AVVQTLVNNNPGK-VPKACCVPTQLDS				
50		80	90	100	
	VAMLYLNDQSTVVLKKNYQEMTVVGCGCR;				

oder

(d)

1 10 20 30 40
 LYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFFPLNS
 50 60 70
 YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
 80 90 100
 ISVLYFDDSSNVILKKYRNMVVRACGCH;

oder

(e)

-5
 HQRQA
 1 10 20 30 40
 CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFFPLNS
 50 60 70
 YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
 80 90 100
 ISVLYFDDSSNVILKKYRNMVVRACGCH;

oder

(f)

1 10 20 30 40
 LYVDFQRDVGWDDWIIAPVDFDAYYCSGACQFPSAD
 50 60 70
 HFNSTN--H-AVVQTLVNNMNPBK-VPKPCCVPTLSA
 80 90 100
 ISMLYLDENSTVVLKNYQEMTVVGCGCR;

oder

(g)

5
1 10 20 30 40
LYVDFQRDVGWDDWIVAPPGYQAFYCSGACQFPSAD
50 60 70
10 HFNSTN--H-AVVQTLVNNMNPBK-VPKPCCVPTLSA
80 90 100
ISMLYLDENEKVVLLKNYQEMVVEGCGCR;

oder

(h)

25 1 10 20 30 40
LYVDFS-DVGWDDWIVAPPGYQAFYCSGACQFPSAD
50 60 70
30 HFNSTN--H-AVVQTLVNNMNPBK-VPKPCCVPTLSA
80 90 100
ISMLYLDENEKVVLLKKYQEMVVEGCGCR.

- 35
9. Protein nach einem der Ansprüche 1 bis 3,
wobei das Protein eine synthetische Polypeptidkette von weniger als 200 Aminosäuren umfasst.
- 40 10. Verfahren nach einem der Ansprüche 4 bis 8,
wobei das Protein eine synthetische Polypeptidkette von weniger als 200 Aminosäuren umfasst.
11. Protein nach Anspruch 3,
wobei die Wirtszelle eine prokaryontische oder eukaryontische Wirtszelle ist, beispielsweise E. coli, Bacillus, Hefen
45 oder Tierzellen (beispielsweise CHO oder Myeloma).
12. Verfahren nach einem der Ansprüche 4 bis 8 oder 10,
wobei die Wirtszelle eine prokaryontische oder eukaryontische Wirtszelle ist, beispielsweise E. coli, Bacillus, Hefen
50 oder Tierzellen (beispielsweise CHO oder Myeloma).
13. Protein nach einem der Ansprüche 1 bis 3,
wobei das Protein unglykosyliert ist.
14. Verfahren nach einem der Ansprüche 4 bis 8 oder 10,
55 wobei das Protein unglykosyliert ist.
15. Zusammensetzung, die ein Protein umfasst, dass zwei Polypeptidketten, die durch Disulfidbrücken verbunden sind, umfasst, um eine dimere Spezies zu erzeugen, wobei das Protein wie in Anspruch 1, 2 oder 3 definiert ist.

16. Osteogene Vorrichtung, die eine Matrix umfasst, in der das Protein nach Anspruch 1, 2 oder 3 angeordnet ist.

17. Osteogene Vorrichtung zur Implantation in ein Säugetier, wobei die Vorrichtung Folgendes umfasst: (a) eine biokompatible, in vivo biologisch abbaubare Matrix, die ein Gerüst mit Abmessungen definiert, die ausreichend sind, um die Anlagerung, Proliferation und Differentiation von migratorischen Vorläuferzellen aus dem Körper des Säugetiers zu ermöglichen, und (b) das Protein nach Anspruch 1, 2 oder 3, das in der Matrix angeordnet und für diese Zellen zugänglich ist.

18. Vorrichtung nach Anspruch 16 oder Anspruch 17, wobei die Matrix eng gepacktes teilchenförmiges Material mit einer Teilchengröße im Bereich von 70 bis 850 µm (beispielsweise 70 bis 420 µm) umfasst.

19. Vorrichtung nach einem der Ansprüche 16 bis 18, wobei die Matrix Folgendes umfasst: (a) allogenen Knochen, beispielsweise demineralisierten, Protein-extrahierten, teilchenförmigen allogenen Knochen, (b) demineralisierten, Protein-extrahierten, teilchenförmigen, deglykosylierten xenogenen Knochen, (c) entmineralisierten, Protein-extrahierten, teilchenförmigen xenogenen Knochen, der mit HF oder einer Protease behandelt ist, (d) Materialien, die aus Kollagen, Hydroxyapatit, Calciumphosphaten (beispielsweise Tricalciumphosphat) und Polymeren ausgewählt sind, die Glycolsäure- und/oder Milchsäure-Monomere umfassen, (e) einen die Form behaltenden Feststoff aus locker haftendem teilchenförmigem Material, beispielsweise Kollagen, (f) einen porösen Feststoff oder (g) ein zerkleinertes Gewebe, beispielsweise Muskelgewebe.

20. Vorrichtung nach einem der Ansprüche 16 bis 19, die in der Markhöhle eines allogenen Knochens angeordnet ist.

21. Vorrichtung nach einem der Ansprüche 16 bis 20 zur Verwendung in der Therapie, beispielsweise zum Induzieren einer lokalen Knorpel- und/oder Ersatzknochen- oder heterotopen Knochenbildung in einem Säugetier durch Implantieren der Vorrichtung in ein Säugetier an einem Ort, der für migratorische Vorläuferzellen zugänglich ist.

22. Vorrichtung nach Anspruch 21 zur Bildung von geformten heterotopen Knochen, wobei die Form des heterotopen Knochens, der gebildet wird, derjenigen der implantierten Vorrichtung entspricht.

23. Verwendung des Proteins nach Anspruch 1, 2 oder 3 zur Herstellung des Folgenden: (a) einer Vorrichtung nach einem der Ansprüche 16 bis 21, oder (b) eines Medikamentes, wobei das Medikament oder die Vorrichtung zur Induktion einer lokalen Knorpel- und/oder Ersatzknochen- oder heterotopen Knochen-Bildung in einem Säugetier vorgesehen ist, indem das Medikament oder die Vorrichtung einem Säugetier an einem Ort eingebracht wird, der für migratorische Vorläuferzellen zugänglich ist, zur periodontalen Behandlung, Knorpelinstandsetzung und zur Behandlung der Osteoarthritis und zur Korrektur von nichtverheilenden Frakturen, erworbenen oder angeborenen kraniofazialen oder anderen Skelettoder dentalen Anomalien.

24. Verwendung nach Anspruch 23 zur Bildung eines geformten heterotopen Knochens, wobei die Form des heterotopen Knochens, der gebildet wird, derjenigen der implantierten Vorrichtung entspricht.

25. Synthetisches Nukleinsäuremolekül zur Verwendung im Verfahren nach einem der Ansprüche 4 bis 8.

26. Wirtszelle zur Verwendung im Verfahren nach einem der Ansprüche 4 bis 8, die das synthetische Nukleinsäuremolekül nach Anspruch 25 enthält.

27. Wirtszelle nach Anspruch 26, die eine prokaryontische oder eukaryontische Wirtszelle ist, beispielsweise E. coli, Bacillus, Hefen oder Tierzellen (beispielsweise CHO oder Myeloma).

28. Isolierte DNA-Sequenz, die die Aminosäuresequenz des wie in einem der Ansprüche bis 3 oder 9 definierten Proteins kodiert.

Revendications

1. Protéine ostéogène comprenant une paire de chaînes polypeptidiques liées à l'état non réduit pour former une

espèce homodimère ou hétérodimère dont la conformation est telle que la paire de chaînes polypeptidiques est capable d'induire une ostéogenèse cartilagineuse lorsqu'elle est disposée à l'intérieur d'une matrice et implantée dans un mammifère, dans laquelle la protéine comprend une région contenant six résidus de cystéine disposés dans les positions relatives représentées dans la séquence :

**LXVFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXXNHAXXQXXVX
XXNXXXXPXXCCXPXXXXXXXXXXLXXXXXXXXVXLXXYXXMXVXXCXCX,**

séquence dans laquelle les lettres indiquent les résidus d'acides aminés d'un code standard à lettre unique et chaque X représente n'importe quel acide aminé, avec cette réserve que l'on exclut le dpp.

2. Protéine selon la revendication 1, dans laquelle la protéine comprend une région contenant sept résidus de cystéine disposés dans les positions relatives représentées dans la séquence :

**CXXXXLXVFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXXNHAXX
QXXVXXXNXXXXPXXCCXPXXXXXXXXXXLXXXXXXXXVXLXXYXXMXVXXCXCX.**

3. Protéine selon la revendication 1 ou 2, à savoir une protéine recombinante que l'on obtient par expression dans une cellule hôte.

4. Procédé pour produire une protéine ostéogène synthétique active, comme défini à la revendication 1, ladite protéine comprenant une paire de chaînes polypeptidiques liées via des ponts disulfure pour produire une espèce dimère, le procédé comprenant les étapes consistant à :

(a) produire une séquence consensus d'acides aminés en comparant des séquences d'acides aminés de protéines de la famille génique TGF-bêta, ladite séquence consensus conservant le modèle de réticulation via des ponts disulfure présent dans la séquence :

(i) **LXVFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXX
HAXXQXXVXXXNXXXXPXXCCXPXXXXXXXXXXLXXXXXXXXVXL
XXYXXMXVXXCXCX;**

ou

(ii) **CXXXXLXVFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXX
XXXXNHAXXQXXVXXXNXXXXPXXCCXPXXXXXXXXXXLXXXX
XXVXLXXYXXMXVXXCXCX;**

séquence dans laquelle les lettres indiquent les résidus d'acides aminés d'un code standard à lettre unique et chaque X représente n'importe quel acide aminé ;

(b) construire un gène de synthèse encodant la séquence consensus d'acides aminés de l'étape (a) par exemple en assemblant deux oligonucléotides qui ont été synthétisés par voie chimique ;

(c) introduire le gène de synthèse obtenu par l'étape (b) dans une cellule hôte procaryote ou eucaryote ;

(d) exprimer le gène de synthèse pour produire une protéine ostéogène synthétique généralement admise ;

(e) analyser la protéine ostéogène synthétique généralement admise dans le but de déterminer le fait de savoir si elle est active en ce qui concerne l'induction d'une ostéogénèse cartilagineuse lors de son implantation dans un mammifère, et le cas échéant

(f) répéter les étapes (a) à (e) (ou procéder à une mutagenèse du gène de synthèse et répéter les étapes (c) à (e) lorsque la protéine ostéogène synthétique généralement admise se révèle inactive lors de l'analyse.

5. Procédé selon la revendication 4, dans lequel la séquence consensus d'acides nucléiques comprend la séquence :

(a) **LXVXFDXGWXXWXXXPXGXAXYCXGXCXXPXXXXXXXXXN
HAXXQXXVXXXNXXXXPXXCCXPXXXXXXXXXXLXXXXXXXXVXL
XXYXXMXVXXCXCX;**

ou

(b) **CXXXXLXVXFDXGWXXWXXXPXGXAXYCXGXCXXPXXXX
XXXXNHAXXQXXVXXNXXXXPXXCCXPXXXXXXXXXXLXXXXX
XXVXLXXYXXMXVXXCXCX;**

séquence dans laquelle les lettres indiquent les résidus d'acides aminés d'un code standard à lettre unique et chaque X représente n'importe quel acide aminé.

6. Procédé selon l'une quelconque des revendications 4 à 5, dans lequel le gène de synthèse de l'étape (b) comprend une séquence nucléotidique qui encode une chaîne polypeptidique synthétique qui possède le même nombre de résidus de cystéine dans les mêmes positions relatives que COP5, COP7, COP16 ou OP1.

7. Procédé selon l'une quelconque des revendications 4 à 6, dans lequel le gène de synthèse de l'étape (b) comprend une séquence nucléotidique qui encode une chaîne polypeptidique synthétique qui partage un ou plusieurs acides aminés à des positions correspondantes dans l'une quelconque des séquences : Vg1, DPP, OP1, CBMP-2a, CBMP-2b, CBMP-3, COP1, COP3, COP4, COP5, COP7 ou COP16.

8. Procédé selon l'une quelconque des revendications 4 à 7, dans lequel le gène de synthèse de l'étape (b) comprend une séquence nucléotidique qui encode une chaîne polypeptidique synthétique comprenant la séquence d'acides aminés :

10 20 30 40 50
 CKRHPLYVDFRDVGWNDWIVPPGYHAFYCHGECPPFLADHLNSTNHAIV
 5 RKRS K S S L QE VIS E FD Y E A AY MPESMKAS VI
 KE F E K I DN L N S Q ITK F P TL
 Q A S K
 60 70 80 90 100
 QTLVNSVNP GKIPKACCVPTELSAISMLYLDENENVVLKRYQDMVVEGCGCR
 10 SI HAI SEQV EP A EQMNSLAI FFNDQDK I RK EE T DA H H
 RF T S K DPV V Y N S H RN RS
 N S K P E

15

dans laquelle, dans chaque position dans laquelle on représente plus d'un acide aminé, l'un quelconque des acides
 aminés représentés peut se trouver dans cette position ; ou

20

10 20 30 40 50
 LYVDFRDVGWNDWIVAPPGYHAFYCHGECPPFLADHLNSTNHAIV
 25 K S S L QE VIS E FD Y E A AY MPESMKAS VI
 F E K I DN L N S Q ITK F P TL
 A S K
 60 70 80 90 100
 QTLVNSVNP GKIPKACCVPTELSAISMLYLDENENVVLKRYQDMVVEGCGCR
 30 SI HAI SEQV EP A EQMNSLAI FFNDQDK I RK EE T DA H H
 RF T S K DPV V Y N S H RN RS
 N S K P E

35

dans laquelle, dans chaque position dans laquelle on représente plus d'un acide aminé, l'un quelconque des acides
 aminés représentés peut se trouver dans cette position ; ou

40

(c)

1 10 20 30 40
 CRRHSLYVDFS-DVGWDDWIVAPLGYDAYYCHGKCPFFPLAD
 45 50 60 70
 HFNSTN--H-AVVQTLVNNNNP GK-VPKACCVPTQLDS
 80 90 100
 50 VAMLYLNDQSTVVLKRYQEMTVVGGCGCR;

ou

55

ou
ou
ou

(e)

(f)

```

1          10          20          30          40
  LYVDFQRDVGWDDWIIAPVDFDAYYCSGACQFPFSAD
          50          60          70
HFNSTN--H-AVVQTLVNNMNP GK-VPKPCCVPTLSA
          80          90         100
ISMLYLDENSTVVLKNYQEMTVVGCGR;

```

(g)

1 10 20 30 40
 LYVDFQRDVGWDDWIVAPPGYQAFYCSGACQFPSAD
 50 60 70
 HFNSTN--H-AVVQTLVNNMNPBK-VPRPCCVPTLSA
 80 90 100
 ISMLYLDENEKVVLLKRYQEMVVEGCGCR;

ou

(h)

1 10 20 30 40
 LYVDFS-DVGWDDWIVAPPGYQAFYCSGACQFPSAD
 50 60 70
 HFNSTN--H-AVVQTLVNNMNPBK-VPRPCCVPTLSA
 80 90 100
 ISMLYLDENEKVVLLKRYQEMVVEGCGCR.

9. Protéine selon l'une quelconque des revendications 1 à 3, dans laquelle la protéine comprend une chaîne polypeptidique synthétique contenant moins de 200 acides aminés.
10. Protéine selon l'une quelconque des revendications 4 à 8, dans laquelle la protéine comprend une chaîne polypeptidique synthétique contenant moins de 200 acides aminés.
11. Protéine selon la revendication 3, dans laquelle la cellule hôte est une cellule hôte procaryote ou eucaryote, par exemple des cellules de E. coli, de Bacillus, de levure ou des cellules animales (par exemple des cellules CHO ou des cellules de myélome).
12. Procédé selon l'une quelconque des revendications 4 à 8 ou 10 dans lequel la cellule hôte est une cellule hôte procaryote ou eucaryote par exemple des cellules de E. coli, de Bacillus, de levure ou des cellules animales (par exemple des cellules CHO ou des cellules de myélome).
13. Protéine selon l'une quelconque des revendications 1 à 3, dans laquelle la protéine est non glycosylée.
14. Procédé selon l'une quelconque des revendications 4 à 8 ou 10 dans lequel la protéine est non glycosylée.
15. Composition comprenant une protéine comprenant une paire de chaînes polypeptidiques liées via des ponts disulfure pour produire une espèce dimère, la protéine étant telle que définie à la revendication 1, 2 ou 3.
16. Dispositif ostéogène comprenant une matrice dans laquelle est disposée la protéine selon la revendication 1, 2 ou 3.
17. Dispositif ostéogène à des fins d'implantation dans un mammifère, le dispositif comprenant : (a) une matrice biocompatible, biodégradable in vivo définissant un support de dimension suffisante pour permettre la fixation, la

prolifération et la différenciation de cellules souches migratrices à partir du corps dudit mammifère, et (b) la protéine selon la revendication 1, 2 ou 3 disposée dans ladite matrice et accessible auxdites cellules.

- 5 18. Dispositif selon la revendication 16 ou 17, dans lequel ladite matrice comprend une matière particulaire fortement densifiée qui possède une granulométrie dans la plage de 70 à 850 μm (par exemple de 70 à 420 μm).
- 10 19. Dispositif selon l'une quelconque des revendications 16 à 18, dans lequel ladite matrice comprend : (a) de l'os allogène, par exemple de l'os allogène, particulaire, extrait d'une protéine, déminéralisé ; (b) de l'os xénogène déglycosylé, particulaire, extrait d'une protéine, déminéralisé ; (c) de l'os xénogène déglycosylé, particulaire, extrait d'une protéine, déminéralisé, qui a été traité avec du HF ou avec une protéase ; (d) de matière choisie parmi le groupe comprenant le collagène, l'hydroxy-apatite, des phosphates de calcium (par exemple le phosphate tricalcique) et des polymères comprenant des monomères d'acide glycolique et/ou d'acide lactique ; (e) un produit solide conservant sa forme, constituée d'une matière particulaire présentant une adhérence lâche, par exemple du collagène ; (f) un produit solide poreux ; ou (g) un tissu masticateur, par exemple un muscle.
- 15 20. Dispositif selon l'une quelconque des revendications 16 à 19, placé dans la cavité médullaire d'os allogène.
- 20 21. Dispositif selon l'une quelconque des revendications 16 à 20, à utiliser en thérapie, par exemple pour induire une formation locale de cartilage et/ou d'os enchondral ou hétérotope chez un mammifère par implantation du dispositif dans un mammifère à un site accessible à des cellules souches migratrices.
- 25 22. Dispositif selon la revendication 21, pour la formation d'os hétérotope configuré, dans lequel la configuration de l'os hétérotope à l'état formé épouse celle du dispositif implanté.
- 30 23. Utilisation de la protéine selon la revendication 1, 2 ou 3 pour la fabrication : (a) d'un dispositif selon l'une quelconque des revendications 16 à 21 ou (b) d'un médicament, le médicament ou le dispositif étant destiné à induire une formation locale de cartilage et/ou d'os enchondral ou hétérotope chez un mammifère en introduisant le médicament ou le dispositif dans un mammifère à un site accessible à des cellules souches migratrices pour un traitement parodontal, une réparation de cartilage et pour le traitement de l'arthrose ou encore pour corriger des fractures en l'absence de soudure, des anomalies craniofaciales acquises ou congénitales, et d'autres anomalies squelettiques ou dentaires.
- 35 24. Utilisation selon la revendication 23 pour la formation d'os hétérotope configuré, dans lequel la configuration de l'os hétérotope à l'état formé épouse celle du dispositif implanté.
- 40 25. Molécule synthétique d'acide nucléique à utiliser dans le procédé selon l'une quelconque des revendications 4 à 8.
- 45 26. Cellule hôte à utiliser dans le procédé selon l'une quelconque des revendications 4 à 8, qui contient la molécule synthétique d'acide nucléique de la revendication 25.
- 50 27. Cellule hôte selon la revendication 26, à savoir une cellule hôte procaryote ou eucaryote, par exemple des cellules de *E. coli*, de *Bacillus*, de levure ou des cellules animales (par exemple des cellules CHO ou des cellules de myélome).
- 55 28. Séquence d'ADN isolée qui encode la séquence d'acides aminés de la protéine telle que définie dans l'une quelconque des revendications 1 - 3 ou 9.

Y,f,n	Y,f,n	C	e,h,*	g	e,a*	C	p,a,g,q	f,y	P	l,m,i	p,a,s,t	d,e,s,g*	h,r,n, s*	m,l,f,p,sa	n,k	s,a,p,g	t,s,a	n	h	a,t,s	i,v,t	v,i,l	q,n	t, s,a,g	l,i	v	h,n r			
:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:			
H	Y	C	H	G	G	C	G	L	H	I	P	P	N	L	S	L	P	V	P	G	A	P	P	T	P	A	Q	P	Y	
N	N	C	Q	G	V	C	G	W	P	Q	S	D	R	N	P	R	Y	G	N	H	V	V	L	L	L	K	M			
:	:	:	A	S	:	:	:	L	:	:	:	:	:	A	:	:	:	:	:	:	:	:	:	:	:	:	:	:		
N	F	C	L	G	P	C	P	Y	I	W	S	L	S	A	:	:	:	:	:	:	:	:	:	:	:	:	:	:		
N	Y	C	E	G	S	C	P	A	Y	L	A	G	V	P	G	S	A	S	S	F	H	T	A	V	V	N	Q	Y	R	M
N	Y	C	E	G	E	C	P	S	H	I	A	G	T	S	G	S	S	L	S	F	H	S	T	V	I	N	H	Y	R	M
Y	Y	C	S	G	A	C	Q	F	P	M	P	K	S	L	K	P	S	N	H	A	T	I	Q	S	I	V	R			
:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:		
F	Y	C	H	G	E	C	P	F	P	L	A	D	H	L	N	S	T	N	H	A	I	V	Q	T	L	V	N			
Y	Y	C	E	G	E	C	A	F	P	L	N	S	Y	M	N	A	T	N	H	A	I	V	Q	T	L	V	H			
Y	Y	C	H	G	K	C	P	F	P	L	A	D	H	F	N	S	T	N	H	A	V	V	Q	T	L	V	N			
N	Y	C	Y	G	E	C	P	Y	P	L	T	E	I	L	N	G	S	N	H	A	I	L	Q	T	L	V	H			
:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:			
F	Y	C	H	G	E	C	P	F	P	L	A	D	H	L	N	S	T	N	H	A	V	V	Q	T	L	V	N			
F	Y	C	H	G	E	C	P	F	P	L	A	D	H	F	N	S	T	N	H	A	V	V	Q	T	L	V	N			
F	Y	C	S	G	A	C	Q	F	P	S	A	D	H	F	N	S	T	N	H	A	V	V	Q	T	L	V	N			
F	Y	C	S	G	A	C	Q	F	P	S	A	D	H	F	N	S	T	N	H	A	V	V	Q	T	L	V	N			
Y	Y	C	S	G	A	C	Q	F	P	S	A	D	H	F	N	S	T	N	H	A	V	V	Q	T	L	V	N			

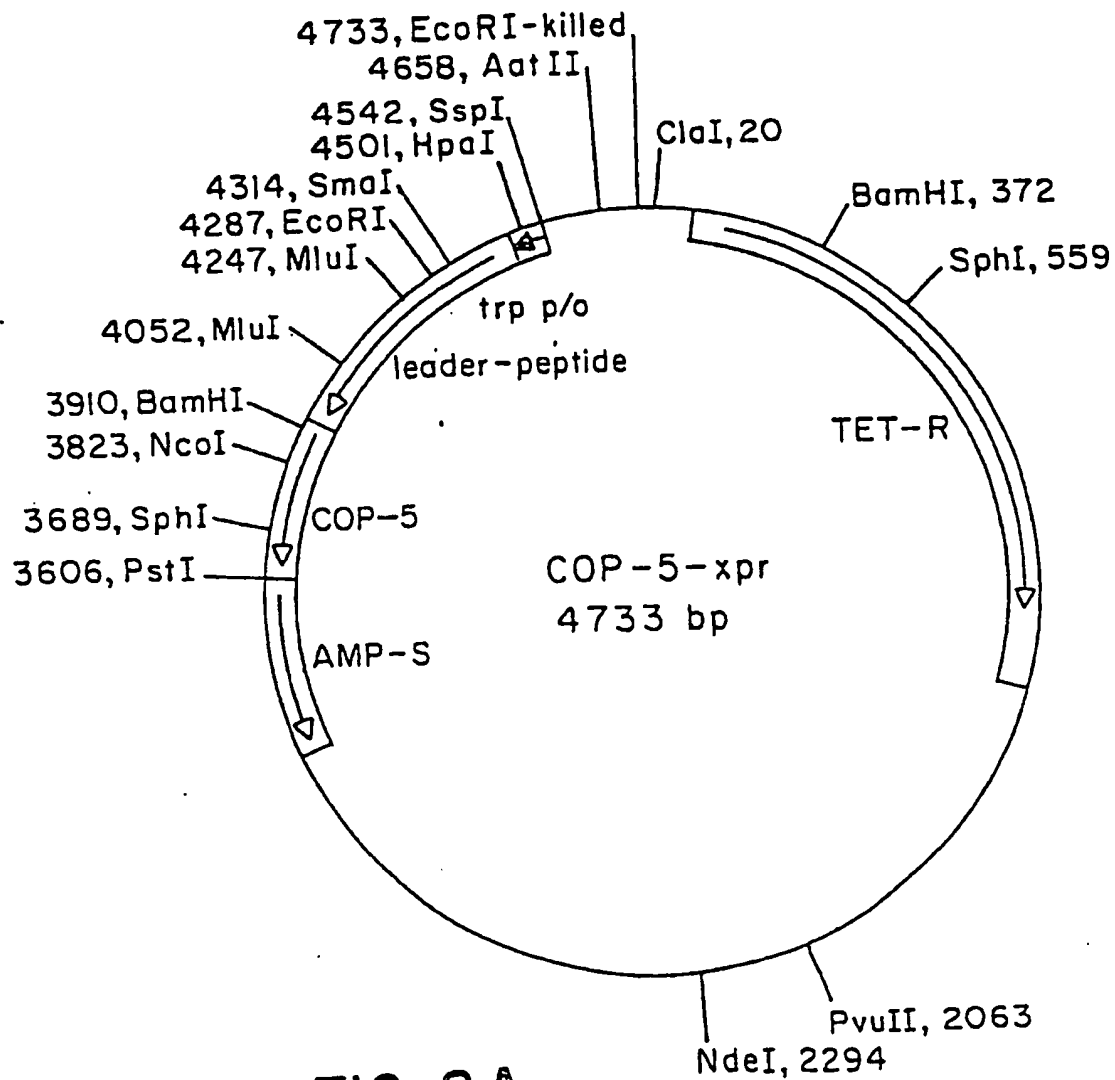


FIG. 2A

FIG. 2B-1

COP-5 fusion protein

```

      10      20      30      40      50
ATGAAAGCAATTTTCGTACTGAAAGGTTCACTGGACAGAGATCTGGACTC
M K A I F V L K G S L D R D L D S
                               BglIII

      60      70      80      90      100
TCGTCTGGATCTGGACGTTTCGTACCGACCACAAAGACCTGTCTGATCACC
R L D L D V R T D H K D L S D H

      110     120     130     140     150
TGGTTCTGGTCGACCTGGCTCGTAACGACCTGGCTCGTATCGTTACTCCC
L V L V D L A R N D L A R I V T P
      SalI                               Sma

      160     170     180     190     200
GGGTCTCGTTACGTTGCGGATCTGGAATTCATGGCTGACAACAAATTCAA
G S R Y V A D L E F M A D N K F N
I                               EcoRI

      210     220     230     240     250
CAAGGAACAGCAGAACGCGTTCTACGAGATCTTGCACCTGCCGAACCTGA
K E Q Q N A F Y E I L H L P N L
              MluI           BglIII       BspMI+

      260     270     280     290     300
ACGAAGAGCAGCGTAACGGCTTCATCCAAAGCTTGAAGGATGAGCCCTCT
N E E Q R N G F I Q S L K D E P S
                               HindIII

      310     320     330     340     350
CAGTCTGCCAATCTGCTAGCGGATGCCAAGAACTGAACGATGCGCAGGC
Q S A N L L A D A K K L N D A Q A
              NheI                               PspI

      360     370     380     390     400
ACCGAAATCGGATCAGGGGCAATTCATGGCTGACAACAAATTCAACAAGG
P K S D Q G Q F M A D N K F N K

      410     420     430     440     450
AACAGCAGAACGCGTTCTACGAGATCTTGCACCTGCCGAACCTGAACGAA
E Q Q N A F Y E I L H L P N L N E
      MluI           BglIII       BspMI+

      460     470     480     490     500
GAGCAGCGTAACGGCTTCATCCAAAGCTTGAAGGATGAGCCCTCTCAGTC
E Q R N G F I Q S L K D E P S Q S
                               HindIII

```

FIG. 2B-2

510 520 530 540 550
 TCGGAATCTGCTAGCGGATGCCAAGAACTGAACGATGCGCAGGCACCGA
 A N L L A D A K K L N D A Q A P
 NheI FspI

560 570 580 590 600
 AGGATCCTAATGGGCTGTACGTCGACTTCAGCGACGTGGGCTGGGACGAC
 K D P N G L Y V D F S D V G W D D
 BamHI Sall

610 620 630 640 650
 TGGATTGTGGCCCCACCAGGCTACCAGGCCTTCTACTGCCATGGCGAATG
 W I V A P P G Y Q A F Y C H G E C
 StuI NcoI BsmI+

660 670 680 690 700
 CCCTTTCCCGCTAGCGGATCACTTCAACAGCACCAACCACGCCGTGGTGC
 P F P L A D H F N S T N H A V V
 NheI DraIII
 PflMI

710 720 730 740 750
 AGACCCTGGTGAACCTCTGTCAACTCCAAGATCCCTAAGGCTTGCTGCGTG
 Q T L V N S V N S K I P K A C C V
 MstII

760 770 780 790 800
 CCCACCGAGCTGTCCGCCATCAGCATGCTGTACCTGGACGAGAATGAGAA
 P T E L S A I S M L Y L D E N E K
 SphI

810 820 830 840 850
 GGTGGTGCTGAAGAACTACCAGGAGATGGTAGTAGAGGGCTGCGGCTGCC
 V V L K N Y Q E M V V E G C G C
 PflMI

860
 GCTAACTGCAG
 R. *
 PstI

FIG. 3A

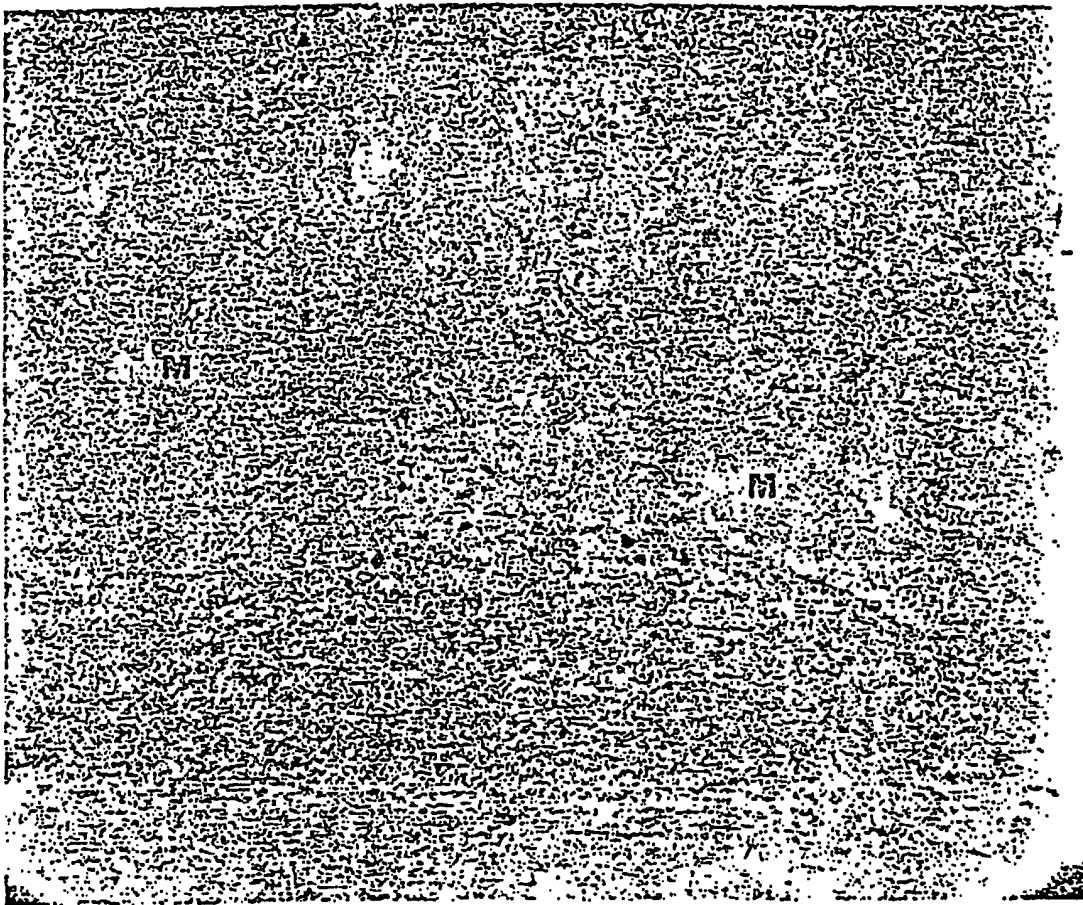


FIG. 3B



FIG. 4

10 20 30 40 50
GATCCTAATGGGCTGTACGTGGACTTCCAGCGCGACGTGGGCTGGGACGA
D P N G L Y V D F Q R D V G W D D

60 70 80 90 100
CTGGATCATCGCCCCCGTCGACTTCGACGCCTACTACTGCTCCGGAGCCT
W I I A P V D F D A Y Y C S G A

110 120 130 140 150
GCCAGTTCCCCTCTGCGGATCACTTCAACAGCACCAACCACGCCGTGGTG
C Q F P S A D H F N S T N H A V V

160 170 180 190 200
CAGACCCTGGTGAACAACATGAACCCCGGCAAGGTACCCAAGCCCTGCTG
Q T L V N N M N P G K V P K P C C

210 220 230 240 250
CGTGCCCAACCGAGCTGTCCGCCATCAGCATGCTGTACCTGGACGAGAATT
V P T E L S A I S M L Y L D E N

260 270 280 290 300
CCACCGTGCTGCTGAAGAACTACCAGGAGATGACCGTGCTGGGCTGCGGC
S T V V L K N Y Q E M T V V G C G

310
TGCCGCTAACTGCAG
C R *

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